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(54) Title: DOPAMINE RECEPTOR PEPTIDES AND ANTI-PEPTIDE ANTIBODIES			
(57) Abstract Dopamine receptor peptide analogues corresponding to various regions of difference in the D ₁ -D ₅ receptors as well as anti-peptide antibodies elicited by the peptide analogues, which are useful in the drug research and disease diagnostic fields.			
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DOPAMINE RECEPTOR PEPTIDES AND ANTI-PEPTIDE ANTIBODIES

BACKGROUND OF THE INVENTION

The present invention relates to synthetic peptide analogues to regions of difference in dopamine receptors and to the anti-peptide antibodies which can be elicited with the peptide analogues.

Dopamine is a neurotransmitter in the central nervous system of the mammalian brain. Dopamine is believed to be involved in the regulation of a variety of functions including motor coordination, emotional stability, and reproduction regulation.

Five (5) different sub-types of dopamine receptors have been mapped and described. These receptors have been classified into two (2) major families: the D₁ family which includes the D₁ and D₅ receptors, and the D₂ family which includes the D₂, D₃, and D₄ receptors.

All dopamine receptors are members of the G protein-coupled receptor gene superfamily, each having seven transmembrane spanning domains. In addition to the five sub-types of dopamine receptors, the D₂ receptor exists in two distinct forms (the long and short forms) which differ in the presence or absence of a 29 amino acid long segment. This difference is due to alternative splicing of the mRNA.

Each of the distinct forms of the dopamine receptor possesses high sequence homology to all the other receptor forms. The occurrence of high sequence homology has made the development of isoform specific antibodies difficult in the past, if not nearly impossible, by the use of the individual receptors themselves.

Farooqui, S.M., et al. [Journal of Neurochemistry, Vol 57, No. 4 (1991) 1363-1369] have reported the use of two synthetic peptides to develop antibodies which recognize the D₂ receptor in rat striatum. The two peptides reported correspond to amino acids #24-34 and #176-185 of the rat D₂ receptor.

Boundy, V. A., et al. [Journal of Neurochemistry,

Vol 60, No. 6 (1993) 2181-2191] have reported the use of fusion proteins to develop antibodies for the D₂ receptors and for the short form of the D₂ receptor.

Unfortunately, the prior progress in this field has been severely limited in that biochemical reagents for all the isoforms have not been ascertained, or those that have lack specificity with respect to the long and short forms of the D₂ receptor.

SUMMARY OF THE INVENTION

The present invention provides for synthetic peptide analogues to regions of difference of the dopamine receptors and anti-peptide antibodies elicited thereby. Peptide analogues to various regions of difference of dopamine receptors were constructed.

In addition, the peptide analogues have been used to elicit antibodies after conjugation onto carrier proteins. Each antiserum obtained from rabbits was tested by ELISA for the production of antibodies specific for the synthetic peptide analogue used as the immunogen. The antisera resulting positively were tested for their ability to recognize mature whole protein in ELISA's and the denatured whole protein in SDS-PAGE/western immunoblots in whole rat brain homogenate, whole mouse brain homogenate, PC-12 cell lysate, and NRK cell lysate.

Further, the antisera were also tested for their ability to immunostain fixed PC-12 cells and fixed NRK cells. Specificity was assessed by the ability of the immunogen peptide to block the anti-peptide antibody binding in these various assays.

It is therefore an object of the present invention to provide a series of peptides which correspond to various regions of difference in the D₁-D₅ receptors of dopamine in order to develop a panel of antibodies specific for the individual dopamine receptor isoforms.

It is another object of the present invention to develop synthetic peptide analogues to regions of difference of the dopamine receptors for use as tools by drug discovery programs and investigators involved in basic research.

Another object of the present invention is to provide dopamine receptor isoform specific anti-peptide antibodies specific for the regions of difference in the dopamine receptors which will be employed as diagnostic entities for disease states such as Parkinson's disease.

A further object of the present invention is to

provide dopamine receptor peptides peculiar to the D_1 - D_5 receptors of dopamine which may be employed in blocking experiments to show specificity.

The invention possesses other objects and advantages which will become apparent as the specification continues.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 (parts A-E) shows the amino acid sequences for peptides which are analogues for the D₁ dopamine receptor isoform.

FIG. 2 (parts A-J) shows the amino acid sequences for peptides which are analogues for the D₂ dopamine receptor isoform.

FIG. 3 (parts A-D) shows the amino acid sequences for peptides which are analogues for the D₃ dopamine receptor isoform.

FIG. 4 (parts A-F) shows the amino acid sequences for peptides which are analogues for the D₄ dopamine receptor isoform.

FIG. 5 (parts A-E) shows the amino acid sequences for peptides which are analogues for the D₅ dopamine receptor isoform.

FIG. 6 is a graphical representation of the ELISA titration of five dopamine D₁ receptor anti-peptide antisera for Rat Brain Homogenate at 200 ngm/well.

FIG. 7 is a graphical representation of the ELISA titration of six dopamine D₂ receptor anti-peptide antisera for Rat Brain Homogenate at 200 ngm/well.

FIG. 8 is a graphical representation of the ELISA titration of four dopamine D₃ receptor anti-peptide antisera for Rat Brain Homogenate at 200 ngm/well.

FIG. 9 is a graphical representation of the ELISA titration of four dopamine D₄ receptor anti-peptide antisera for Rat Brain Homogenate at 200 ngm/well.

FIG. 10 is a graphical representation of the ELISA titration of six dopamine D₅ receptor anti-peptide antisera for Rat Brain Homogenate at 200 ngm/well.

FIG. 11 is a graphical representation of the ELISA titration of five dopamine D_{2L} receptor anti-peptide antisera for Rat Brain Homogenate at 200 ngm/well.

FIG. 12 is a graphical representation of the ELISA titration of the peptide D_{2L} (243-254) cyclized antiserum for four different tissue and cell preparations

at 200 ngm protein/well.

FIG. 13 is a graphical representation of the ELISA titration of the peptide D₂ (Ac175-182) antiserum for four different tissue and cell preparations at 200 ngm protein/well.

FIG. 14 is a graphical representation of the ELISA titration of the peptide D₅ (Ac23-35Cys³⁶) antiserum for four different tissue and cell preparations at 200 ngm protein/well.

FIG. 15 is a graphical representation of the ELISA titration of the peptide D₃ (Ac209-217) antiserum for four different tissue and cell preparations at 200 ngm protein/well.

FIGS. 16 A-O are photographs showing the indirect immunofluorescent staining of specific dopamine receptor isoforms by certain anti-peptide antibodies identified as numbered in Table II, in conjunction with FITC conjugated second antibody, described in Example 7.

FIG. 17 is a photograph of an SDS-PAGE/western immunoblot of PC-12 cell lysate with one of the D₂, D₃, and D₅ isoform specific anti-peptide antibodies as described in Example 6.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Various aspects of the present invention will evolve from the following detailed description of the preferred embodiments thereof which should be referenced to the prior described drawings.

Published amino acid sequences for each of the five dopamine receptor isoforms are known and these amino acid sequences were employed in the chemical design of the peptides of the present invention. By way of illustration, Civelli et al., European Journal of Pharmacology - Molecular Pharmacology Section, Vol. 207 (1991) 277-286; Grandy et al., Proc. National Academy of Science, U.S.A., Vol. 88 (1991) 9175-9179; Sibley et al., TIPS Reviews, Vol. 13 (1992) 61-69; Van Tol et al., Nature, Vol. 350 (1991) 610-614; and Grandy et al., Current Opinion in Neurobiology, Vol. 2. (1992) 275-281 discuss such receptors. Amino acid segments were analyzed to construct peptide analogues which are both receptor specific and which will elicit antibodies. It is anticipated that such antibodies, either polyclonal or monoclonal, will cross-react with the native protein even though the antibodies themselves were raised only against the peptide analogue. It is believed that the necessity of raising antibodies to the regions of difference is dictated by the high degree of sequence homology found in the five different isoforms of the dopamine receptor. Figs. 1-5 represent various synthetic peptide analogues constructed for each of the five isoforms of the dopamine receptor.

Each peptide depicted in Table I was synthesized by solid phase peptide synthesis utilizing the Fmoc protecting strategy. Figs. 1-5 represent the amino acid sequences of Table I. The Ac designation of Table I indicates acetylation of the amino terminus for the purpose of eliminating the charged end group in certain instances. The synthetic peptides were cleaved from the solid support resin, isolated, and purified by standard procedures including preparative HPLC. The peptides were also

analyzed for purity by analytical HPLC and for composition by amino acid analysis.

TABLE I**D₁ Receptor**

Receptor		
<u>Batch #</u>	<u>Fragment</u>	<u>Peptide Sequence</u>
1. PS-3511	(Ac-9-21)	Ac-M-D-G-T-G-L-V-V-E-R-D-F-S
2. PS-3512	(Ac-9-21Cys ²²)	Ac-M-D-G-T-G-L-V-V-E-R-D-F-S-C
3. PS-3513	(2-10)	R-T-L-N-T-S-A-M-D
4. PS-3514	(Ac-6-19Cys ²⁰)	Ac-T-S-A-M-D-G-T-G-L-V-V-E-R-D-C
5. PS-3515	(Ac-165-173Cys ¹⁷⁴)	Ac-K-A-K-P-T-S-P-S-D-C

D₂ Receptor

Receptor		
<u>Batch #</u>	<u>Fragment</u>	<u>Peptide Sequence</u>
6. PS-3520	D _{2s} (Ac-240-247Cys ²⁴⁸)	Ac-P-L-K-E-A-A-R-R-C
7. PS-3521	(272-282)	A-A-R-R-A-Q-E-L-E-M-E
8. PS-3522	(24-34Cys ³⁵)	G-S-D-G-K-A-D-R-P-H-Y-C
9. PS-3523	(Ac-25-34)	Ac-S-D-G-K-A-D-R-P-H-Y
10. PS-3524	D _{2t} (243-254) Cyclized	N-C-T-H-P-E-D-M-K-L-C-T -----S-S-----
11. PS-3525	(Ac-25-34Cys ³⁵)	Ac-S-D-G-K-A-D-R-P-H-Y-C
12. PS-3526	(Cys ²⁷¹ -272-282)	C-A-A-R-R-A-Q-E-L-E-M-E
13. PS-3527	(2-12Cys ¹³)	D-P-L-N-L-S-W-Y-D-D-D-C
14. PS-3528	(Ac-19-32Cys ³³)	Ac-S-R-P-F-N-G-S-D-G-K-A-D-R-P-C
15. PS-3529	(Ac-175-182)	Ac-N-N-A-D-Q-N-E-C

D₃ Receptor

Receptor		
<u>Batch #</u>	<u>Fragment</u>	<u>Peptide Sequence</u>
16. PS-3531	(22-32)	G-A-S-Q-A-R-P-H-A-Y-Y
17. PS-3532	(2-10Cys ¹¹)	A-S-L-S-Q-L-S-S-H-C
18. PS-3533	(Ac-17-29Cys ³⁰)	Ac-A-E-N-S-T-G-A-S-Q-A-R-P-H-C
19. PS-3534	(Ac-173-181)	Ac-N-T-T-G-D-P-T-V-C

D₄ Receptor

Receptor		
<u>Batch #</u>	<u>Fragment</u>	<u>Peptide Sequence</u>
20. PS-3541	(Ac-22-35)	Ac-A-S-A-G-A-S-A-G-L-A-G-Q-G-A
21. PS-3542	(Ac-22-35Cys ³⁶)	Ac-A-S-A-G-A-S-A-G-L-A-G-Q-G-A-C
22. PS-3543	(2-10)	G-N-R-S-T-A-D-A-D

23. PS-3544	(Ac-16-30Cys ³¹)	Ac-R-G-P-A-A-G-A-S-A-G-A-S-A-G-L-C
24. PS-3545	(Ac-176-185)	Ac-D-V-R-G-R-D-P-A-V-C
25. PS-3546	(Ac-186-192Lys ¹⁹³)	Ac-R-L-E-D-R-D-Y-K

D₅ Receptor

Receptor		
<u>Batch #</u>	<u>Fragment</u>	<u>Peptide Sequence</u>
26. PS-3551	(26-40)	A-V-G-G-S-A-G-A-P-P-L-G-P-S-Q
27. PS-3552	(2-11Cys ¹²)	L-P-P-G-S-N-G-T-A-Y-C
28. PS-3553	(Ac-23-35Cys ³⁶)	Ac-Q-G-N-A-V-G-G-S-A-G-A-P-P-C
29. PS-3554	(Ac-181-189Lys ¹⁹⁰)	Ac-H-R-D-Q-A-A-S-W-G-K
30. PS-3555	(Ac-209-217)	Ac-E-P-D-V-N-A-E-N-C

Peptides numbered 1, 2, 4-6, 8-12, 14, 15, 18-21, 23-25, and 28-30 in Table I may be employed in the present application with the amino terminus acetylated or un-acetylated.

In addition, each synthetic peptide of Table I was conjugated onto a carrier protein, either bovine thyroglobulin (thyro) or keyhole limpet hemocyanin (KLH). The peptides were conjugated onto the carrier protein using either the EDAC or sulfo-MBS chemistries to construct the immunogen for the elicitation of antibodies.

The peptide/protein conjugates were used as immunogens in rabbits. The different immunogens were used to immunize groups of 2-3 rabbits each. The rabbits were immunized, boosted, and bled following a standard protocol.

The antiserum obtained from each bleed of each rabbit was tested by ELISA for the production of antibodies specific for the synthetic peptide analogue. Those antisera found positive for production of antibodies specific for the peptide portion of the immunogen were then assessed for their ability to recognize the whole protein. They were analyzed for their ability to recognize the native whole protein in ELISA's and the denatured whole protein in SDS-PAGE/Western immunoblots in PC-12 cell lysate, in NRK cell lysate, in whole rat brain homogenates, and in whole mouse brain homogenate. They were also tested

for their ability to stain fixed PC-12 cells and fixed NRK cells. Specificity was assessed by the ability of the immunogen peptide to block, specifically, the antibody binding in these various assays. Table II represents the detection of dopamine receptor isoforms using these anti-peptide antibodies. The term "Antibody to Fragment" in Table II refers to the anti-peptide antibody raised to the specific peptide immunogen of the peptides of Table I. The peptides of Table I are identified according to dopamine receptor fragments.

TABLE II

<u>Antibody To Fragment</u>	<u>Rat Brain</u>	<u>Mouse Brain</u>	<u>PC-12 Cells</u>	<u>NRK Cells</u>
1. D ₁ receptor (Ac-9-21)	E W	E W	N N N	N N N
2. D ₁ receptor (Ac-9-21Cys ²²)	E W	E W	N N N	N N N
3. D ₁ receptor (2-10)	E W	E W	N N N	N N N
4. D ₁ receptor (Ac-6-19Cys ²⁰)	E W	E W	N N N	N N N
5. D ₁ receptor (Ac-165-173Cys ¹⁷⁴)	E W	E W	N N N	N N N
6. D _{2s} receptor (Ac-240-247Cys ²⁴⁸)	E W	E W	N N N	N N N
7. D ₂ receptor (272-282)	E W	E W	E W F	E W F
8. D ₂ receptor (24-34Cys ³⁵)	E W	E W	E W F	E W F
9. D ₂ receptor (Ac-25-34)	E W	E W	E W F	E W F
10. D _{2l} receptor (243-254) cyclized	E W	E W	E W F	E W F
11. D ₂ receptor (Ac-25-34Cys ³⁵)	E W	E W	E W F	E W F
12. D ₂ receptor (Cys ²⁷¹ -272-282)	E W	E W	E W F	E W F
13. D ₂ receptor (2-12Cys ¹³)	E W	E W	E W F	E W F
14. D ₂ receptor (Ac-19-32Cys ³³)	E W	E W	E W F	E W F
15. D ₂ receptor (Ac-175-182)	E W	E W	E W F	E W F
16. D ₃ receptor (22-32)	E W	E W	E W F	N N N
17. D ₃ receptor (2-10Cys ¹¹)	E W	E W	E W F	N N N
18. D ₃ receptor (Ac-17-29Cys ³⁰)	E W	E W	E W F	N N N
19. D ₃ receptor				

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	(Ac-173-181)	E W	E W	E W F	N N N
20.	D ₄ receptor (Ac-22-35)	E W	E W	N N N	E W F
21.	D ₄ receptor (Ac-22-35Cys ³⁶)	E W	E W	N N N	E W F
22.	D ₄ receptor (2-10)	E W	E W	N N N	E W F
23.	D ₄ receptor (Ac-16-30Cys ³¹)	E W	E W	N N N	E W F
24.	D ₄ receptor (Ac-176-185)	E W	E W	N N N	E W F
25.	D ₄ receptor (Ac-186-192Lys ¹⁹³)	E W	E W	N N N	E W F
26.	D ₅ receptor (26-40)	E W	E W	E W F	E W F
27.	D ₅ receptor (2-11Cys ¹²)	E W	E W	E W F	E W F
28.	D ₅ receptor (Ac-23-35Cys ³⁶)	E W	E W	E W F	E W F
29.	D ₅ receptor (Ac-181-189Lys ¹⁹⁰)	E W	E W	E W F	E W F
30.	D ₅ receptor (Ac-209-217)	E W	E W	E W F	E W F

Where, E = ELISA positive, W = Western immunoblot positive, F = Immunofluorescent staining, and N = negative result. Figs. 6-11 represents plots of the ELISA titration of D₁, D₂, D₃, D₄, and D₅ receptor anti-peptide antiserum at various dilutions for rat brain homogenate at 200 ngm/well. The optical density values were obtained from an ELISA plate reader, SLT Lab instruments, Easy Beam Reader Model, Salzburg, Austria. It may be observed that the positive ELISA response was indicated therein. Figs. 12-15 show ELISA titration data for various antisera obtained from the particular peptide conjugate used as an immunogen as heretofore described. The particular antisera is identified in Table II according to the corresponding receptor fragment named in Table I. These antisera were found to show positive results for rat brain homogenate and mouse brain homogenate. PC-12 cell lysate was determined to contain D₂, D_{2L}, D₃, and D₅ isoforms, and NRK cell lysate was found to contain the D₂, D_{2L}, D₄, and D₅ isoforms (Table II).

The following examples are shown herein to be illustrative of the present invention, but are not deemed to limit the same in any manner.

EXAMPLE 1 - SYNTHESIS OF PEPTIDES

All the peptides of Figs. 1-5 were synthesized at the 0.2 mmole scale by standard Fmoc-Solid Phase Peptide Synthesis (Fmoc-SPPS/HOBt/DIPCDI procedures) using a four fold molar excess of reagents. The sequences of the various peptides for the dopamine D₁-D₅ receptor isoforms listed in Table I indicate the results of such synthesis. Each peptide shown in Table I was cleaved from the resin and the side chains of the trifunctional amino acids were simultaneously deprotected using 25 ml. of trifluoroacetic acid (TFA) which contained an appropriate mixture of scavengers. The spent resin was washed with 100% acetic acid (HOAc), 50% HOAc/50%H₂O, and H₂O. The solutions were pooled, rotary evaporated twice, and the peptide partially purified by gel filtration chromatography on a 200 ml column of Sephadex G-10 washed and equilibrated with 25% HOAc in water. The column was developed with 25% HOAc in water and fractions were collected. The fractions which contained the particular peptide were identified by two qualitative spot tests. The first was developed with ninhydrin and the other with chlorine-tolidine. Fractions which contained the partially purified peptide were pooled. For the cyclized D_{2L} (243-254) peptide, number 10 on Table I, the total volume was adjusted to 800 ml with water. The acetic acid was then neutralized and the pH adjusted to pH 8.1 with dilute NH₄OH. The peptide was air oxidized for three (3) days with constant slow mixing to form the intrachain disulfide bond and to cyclize the peptide. The loss of free SH groups was monitored during the air oxidation by the quantitative DTNB assay. The solution was centrifuged to remove precipitated material and filtered through a 1.0 micron glass filter. The solution was then pumped at 3 ml/min onto a semi-preparative C₁₈ reverse phase HPLC column and the particular peptide eluted from the column with a gradient of increasing acetonitrile. For all other peptides in Table I, the acetic acid was removed by rotary evaporation and the peptide diluted to 40 ml with

distilled water. This solution was pumped onto the semi-preparative C₁₈ reverse phase HPLC column. The eluent from the column was monitored for optical density (OD) at 225 nm and fractions were collected. The fractions which contained the peptide were transferred to pre-weighed vials, lyophilized and analyzed. The overall yield for these various syntheses ranged from 24% to 78% of theoretical yield based upon final weight.

EXAMPLE 2 - CONJUGATION OF PEPTIDES TO BOVINE

THYROGLOBULIN OR KEYHOLE LIMPET HEMOCYANIN

Each of the peptides of Table I was conjugated onto either bovine thyroglobulin or keyhole limpet hemocyanin (KLH) using either EDAC or Sulfo-MBS as the cross linking reagent. Peptides which contained a Cys residue were conjugated using sulfo-MBS and all the others were conjugated using EDAC. The coupling ratios used were 200 molecules of antigenic peptide per molecule of thyroglobulin and 2000 molecules of antigenic peptide per molecule of KLH. Each reaction was performed using standard procedures and routine reaction conditions. The conjugates were isolated from the reaction mixture by gel filtration on Sephadex G-25 and lyophilized from a volatile buffer solution. The yields based upon the weights of the final products ranged from 75% to greater than 95%. The peptide/protein conjugates were then used in conjunction with Freund's complete adjuvant to immunize animals.

EXAMPLE 3 - IMMUNIZATION OF RABBITS

Groups of rabbits were immunized with each one of the peptide/protein immunogens in an oil in water emulsion with Freund's complete adjuvant. All animals followed the same boost/bleed schedule which is detailed below.

<u>Day</u>	<u>Procedure</u>
0	Immunize intramuscularly in the left thigh
7	Immunize intramuscularly in the right thigh
56	Boost subcutaneously at multiple sites on the back
70	Bleed from the central ear artery

Continue 14 day/14 day boost/bleed cycles

**EXAMPLE 4 - SCREENING ELISA OF THE ANTISERUM OBTAINED
FROM EACH ANIMAL USING ANTIGENIC PEPTIDE**

ELISA was used to assess the response obtained in eliciting the production of antibodies by each of the immunogens in each animal of all the test groups. The ELISA conditions used for these assays are as follows:

- A. Sensitize plates overnight with 100 ng of antigenic peptide per well in bicarbonate buffer pH 9.6
- B. Wash twice with PBS/tween
- C. Block for 2 hrs with 0.1% BSA in PBS
- D. Wash twice with PBS/tween
- E. 2 fold serial dilutions of antisera bound overnight
- F. Wash four times with PBS/tween
- G. Affinity purified HRP-goat anti-rabbit IgG 2nd antibody bound for 3 hours
- H. Wash four times with PBS/tween
- I. OPD with H_2O_2 reaction run for 30 min. and stopped with sulfuric acid
- J. Read plates at 492 nm

The assays were set up on 96 well high binding microtiter plates as follows:

- Row A = Serial dilutions of pooled preimmune sera
- Rows B-H = Serial dilutions of the antiserum
obtained from each animal of the group
- Column 1 = Blank (No serum bound to well)
- Columns 2-12 = 2 fold serial dilutions of antiserum
from 1:100 to 1:102,400

**EXAMPLE 5 - ELISA VS. RAT BRAIN HOMOGENATE, MOUSE
BRAIN HOMOGENATE, PC-12 CELL LYSATE AND NRK CELL LYSATE**

Each antiserum which was scored as positive for the production of anti-peptide antibodies was then tested for specific recognition of native proteins in four different preparations: rat brain homogenate, mouse brain homogenate, PC-12 cell lysate, and NRK cell lysate. The

ELISA procedure was similar to that described in Example 4 above for the peptide screening ELISA, except the microtiter plates were sensitized at three different levels of total protein (200 ngm/well, 500 ngm/well and 1 μ gm/well) with one of the four different cellular preparations.

At least one, and often numerous, antiserum from each group of rabbits was found to bind to the rat and mouse brain homogenates and to titer out like an antibody. The binding and titration could be specifically blocked by pre-incubating the antiserum with the antigenic peptide used to elicit the anti-peptide antibodies. Some of the antisera had very high titers (i.e., greater than 1:50,000). When similar ELISA titration experiments were performed using PC-12 and NRK cell lysates a different pattern of response was found. Some of the antisera which were found to react strongly with the rat and mouse brain homogenates, bound very weakly or not at all, even in the 1 μ gm/well experiments. This indicates the absence of that specific isoform of the dopamine receptor in that cell type. Other antisera were found which reacted as strongly in the PC-12 and NRK lysates as in the rat and mouse brain homogenates. This indicates the presence of that dopamine receptor isoform in that cell type. Specifically, the D₂, D_{2L}, D₃, and D₅ isoforms of the dopamine receptor were found in the PC-12 cells, and the D₂, D_{2L}, D₄, and D₅ isoforms were detected in the NRK cells.

EXAMPLE 6 - SDS-PAGE/WESTERN IMMUNOBLOT ANALYSIS

SDS-PAGE/Western immunoblot analysis of the dopamine receptor isoform specific anti-peptide antibodies was performed using rat brain homogenate, mouse brain homogenate, PC-12 cell lysate, NRK cell lysate and the dopamine receptor isoform specific anti-peptide antibodies. The general procedure is detailed below:

A. After SDS-PAGE of either rat brain homogenate, mouse brain homogenate, PC-12 cell lysate, or NRK cell lysate, the proteins were electrophoretically

transferred onto PVDF membranes. The membranes were blocked overnight with evaporated goat milk diluted 1:4 in TBS/Tween 20 buffer and washed twice with TBS/Tween-20 buffer.

B. The rabbit anti-peptide antiserum specific for one of the isoforms of the dopamine receptor was diluted 1:500 with evaporated goat milk diluted 1:4 in TBS/Tween-20 buffer. This was applied to the membrane and allowed to bind for 2 hours before being washed 4 times with TBS/Tween-20.

C. Affinity purified HRP conjugated goat anti-rabbit IgG was diluted 1:5,000 with evaporated goat milk diluted 1:4 in TBS/Tween 20 buffer. This solution was applied to the membrane and allowed to bind for 1 hour before being washed 3 times and then once overnight with TBS/Tween-20.

D. The membrane was developed using the enhanced DAB reaction in phosphate/citrate buffer, pH 5.0.

At least one and often numerous anti-peptide antiserum from each of the test groups was found to bind specifically to a protein with molecular weight of 52-58 kD in the rat and mouse brain homogenates: i.e., a protein with the correct size to be one of the dopamine receptors. The binding could be specifically blocked by pre-incubating the anti-peptide antiserum with the antigenic peptide. Only the D_2 , D_{2L} , D_3 , and D_5 isoforms of the dopamine receptor were detected in PC-12 cell lysates by western immunoblot analysis, Fig. 17, and in the NRK cell lysates, only D_2 , D_{2L} , D_4 , and D_5 isoforms were detected by this procedure.

**EXAMPLE 7 - IMMUNOFLUORESCENT STAINING OF FIXED PC-12
AND NRK CELLS USING THE DOPAMINE RECEPTOR ISOFORM SPECIFIC
ANTI-PEPTIDE ANTIBODIES**

Both fixed PC-12 cells and NRK cells were examined for the presence or absence of the D_1 - D_5 receptors by the methods detailed below using the anti-peptide antisera which had been found to be positive by the

screening ELISA.

- A. The formalin fixed PC-12 cells were premeabilized in PBS, 0.1% Triton X-100 (pH 7.2) for 20 minutes. Since the NRK cells were fixed with acetone, they did not need to be premeabilized.
- B. Block with PBS containing 0.1% Triton X-100 and 2% Normal Goat Serum (NGS) for 30 minutes.
- C. Wash 5 times with PBS, 0.1% Triton X-100.
- D. Apply dopamine receptor isoform specific primary antibody (the anti-peptide antiserum) in PBS containing 0.1% Triton X-100 and 2% NGS: incubate for 2 hours at room temperature.
- E. Wash 5 times with PBS, 0.1% Triton X-100.
- F. Apply affinity purified FITC conjugate goat anti-rabbit IgG in PBS containing 0.1% Triton X-100 and 2% NGS: incubate for 1 hour at room temperature.
- G. Wash 5 times with PBS, 0.1% Triton X-100, quickly rinse twice with distilled water, and air dry.
- H. Mount coverslip in glycerol based mounting medium which contains DABCO.
- I. Observe and photograph the fluorescent staining, if present, using an epi-fluorescent microscope equipped with FITC excitation and emission filters and a 35 mm camera.

On fixed PC-12 cells, staining was only observed with antisera specific for the D_2 , D_{2L} , D_3 , and D_5 isoforms of the dopamine receptor, and the immunofluorescent staining could be specifically blocked by pre-incubating the antiserum with the antigenic peptide used to elicit the antibodies. The staining corresponds to and confirms both the ELISA and SDS-PAGE/western immunoblot data for this

cell type. Similar experiments using fixed NRK cells show the presence of the D₂, D_{2L}, D₄, and D₅ receptors. This again confirms the ELISA and western blotting data for this cell type. Photographs of the indirect immunofluorescent staining are included herewith as Figure 16 A-O.

EXAMPLE 8 - PRODUCTION OF MONOCLONAL ANTIBODIES

In addition to the development of polyclonal antisera in rabbits, monoclonal antibodies to the dopamine receptor isoform specific peptides are developed by standard techniques. Groups of mice are immunized with the same peptide/protein conjugates described in Example 2 by a modification of the immunization protocol described in Example 3. The immunized mice are test bled from the eye orbital vein, and the anti-plasma obtained is screened by the ELISA procedures described in Examples 4 and 5 by changing only the HRP conjugated 2nd antibody, i.e., HRP-goat anti-mouse IgG 2nd antibody is used instead of the HRP-goat anti-rabbit IgG 2nd antibody. The mice found positive for the production of antibodies which recognize both the peptide immunogen and the whole rat brain homogenate are selected for use in the development of monoclonal antibodies. The spleens of these mice are surgically removed using aseptic techniques and the splenocytes isolated. The splenocytes are fused using polyethylene glycol with the mouse myeloma cell line SP2/0-Ag14. The unfused SP2/0-Ag14 cells are killed during the sterile cell culture using the hypoxanthine, aminopterin, and thymidine (HAT) selection process. The hybrids are screened using a modification of the ELISA screening protocols described in Examples 4 and 5 (see above). The cells in the positive wells are cloned by standard cell culture techniques to isolate a hydridoma cell line which is producing and secreting the monoclonal anti-peptide antibody specific for an isoform of the dopamine receptor.

While in the foregoing, embodiments of the present invention have been set forth in considerable detail for the purposes of making a complete disclosure of

the invention, it may be apparent to those of skill in the art that numerous changes may be made in such detail without departing from the spirit and principles of the invention.

WHAT IS CLAIMED IS

1. The purified peptide analogues for the dopamine D₁, D₂, D₃, D₄, and D₅ receptor isoforms shown in Figs. 1, 2, 3, 4 or 5.
2. The antibodies raised to the purified peptide analogues shown in Figs. 1, 2, 3, 4, or 5.
3. The purified acetylated peptide analogues for the dopamine receptor isoforms shown in Figs. 1A, 1B, 1D, 1E, 2A, 2C-G, 2I, 2J, 3C, 3D, 4A, 4B, 4D-F, or 5C-E.
4. The antibodies of claim 2 which are polyclonal.
5. The antibodies of claim 2 which are monoclonal.

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Met Asp Gly Thr Gly Leu Val Val Glu Arg Asp Phe Ser
5 5

FIG. 1A

Met Asp Gly Thr Gly Leu Val Val Glu Arg Asp Phe
5 10
Ser Cys
15

FIG. 1B

Arg Thr Leu Asn Thr Ser Ala Met Asp
5

FIG. 1C

Thr Ser Ala Met Asp Gly Thr Gly Leu Val Val Glu
5 10
Arg Asp Cys
15

FIG. 1D

Lys Ala Lys Pro Thr Ser Pro Ser Asp Cys
5 10

FIG. 1E

Pro Leu Lys Glu Ala Ala Arg Arg Cys
5

FIG. 2A

Ala Ala Arg Arg Ala Gln Glu Leu Glu Met Glu
5 10

FIG. 2B

Gly Ser Asp Gly Lys Ala Asp Arg Pro His Tyr Cys
5 10

FIG. 2C

Ser Asp Gly Lys Ala Asp Arg Pro His Tyr
5 10

FIG. 2D

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Asn	Cys	Thr	His	Pro	Glu	Asp	Met	Lys	Leu	Cys	Thr
				5					10		
						S		S			

FIG. 2E

Ser	Asp	Gly	Lys	Ala	Asp	Arg	Pro	His	Tyr	Cys
				5					10	

FIG. 2F

Cys	Ala	Ala	Arg	Arg	Ala	Gln	Glu	Leu	Glu	Met	Glu
				5					10		

FIG. 2G

Asp	Pro	Leu	Asn	Leu	Ser	Trp	Tyr	Asp	Asp	Asp	Cys
				5					10		

FIG. 2H

Ser	Arg	Pro	Phe	Asn	Gly	Ser	Asp	Gly	Lys	Ala	Asp
				5					10		
Arg	Pro	Cys									
		15									

FIG. 2I

Asn	Asn	Ala	Asp	Gln	Asn	Glu	Cys
				5			

FIG. 2J

Gly	Ala	Ser	Gln	Ala	Arg	Pro	His	Ala	Tyr	Tyr
				5					10	

FIG. 3A

Ala	Ser	Leu	Ser	Gln	Leu	Ser	Ser	His	Cys
				5					10

FIG. 3B

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Ala Glu Asn Ser Thr Gly Ala Ser Gln Ala Arg Pro
5 10
His Cys

FIG. 3C

Asn Thr Thr Gly Asp Pro Thr Val Cys
5

FIG. 3D

Ala Ser Ala Gly Ala Ser Ala Gly Leu Ala Gly Gln
5 10
Gly Ala

FIG. 4A

Ala Ser Ala Gly Ala Ser Ala Gly Leu Ala Gly Gln
5 10
Gly Ala Cys
15

FIG. 4B

Gly Asn Arg Ser Thr Ala Asp Ala Asp
5

FIG. 4C

Arg Gly Pro Ala Ala Gly Ala Ser Ala Gly Ala Ser
5 10
Ala Gly Leu Cys
15

FIG. 4D

Asp Val Arg Gly Arg Asp Pro Ala Val Cys
5 10

FIG. 4E

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Arg Leu Glu Asp Arg Asp Tyr Lys
5

FIG. 4F

Ala Val Gly Gly Ser Ala Gly Ala Pro Pro Leu Gly
5 10
Pro Ser Gln
15

FIG. 5A

Leu Pro Pro Gly Ser Asn Gly Thr Ala Tyr Cys
5 10

FIG. 5B

Gln Gly Asn Ala Val Gly Gly Ser Ala Gly Ala Pro
5 10
Pro Cys

FIG. 5C

His Arg Asp Gln Ala Ala Ser Trp Gly Lys
5 10

FIG. 5D

Glu Pro Asp Val Asn Ala Glu Asn Cys
5

FIG. 5E

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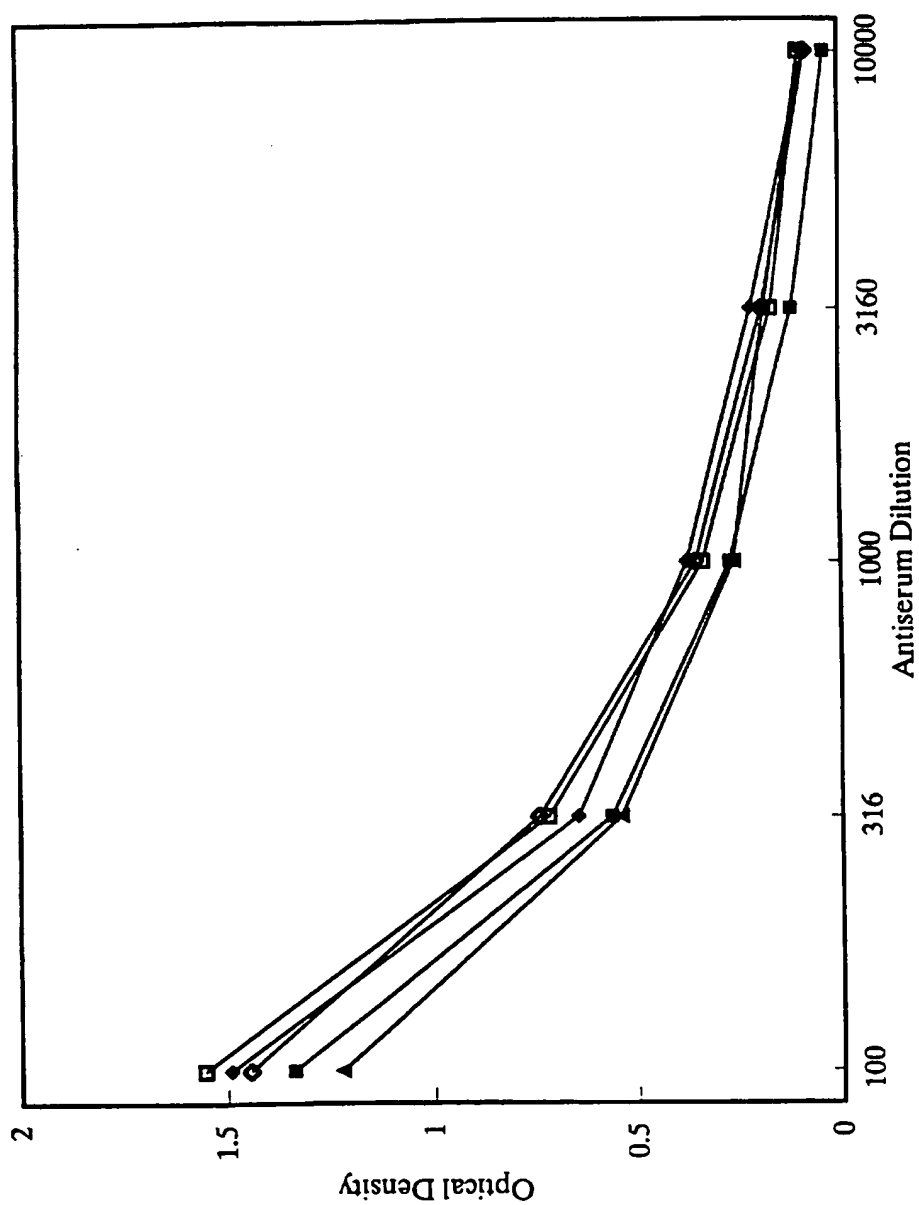


Fig. 6

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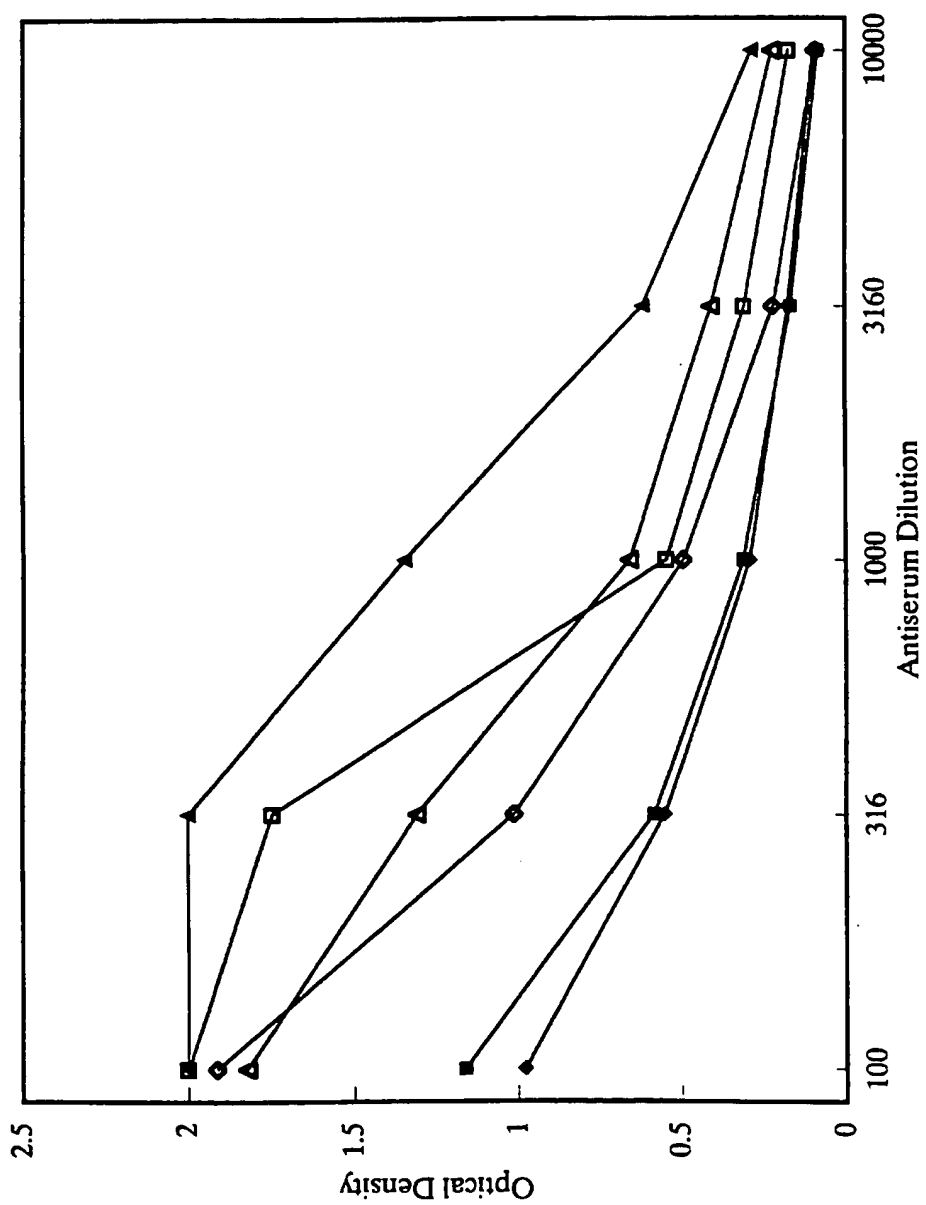


Fig. 7

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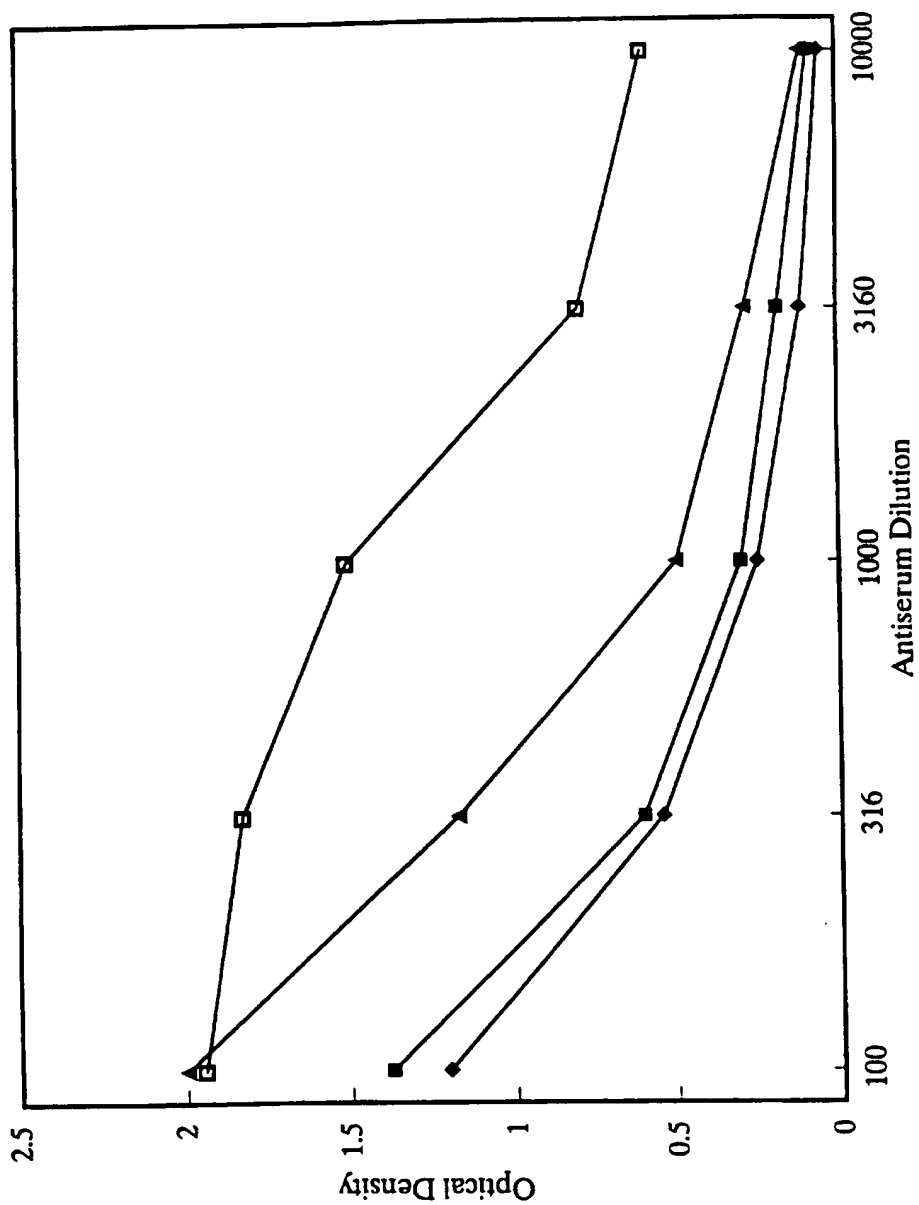


Fig. 8

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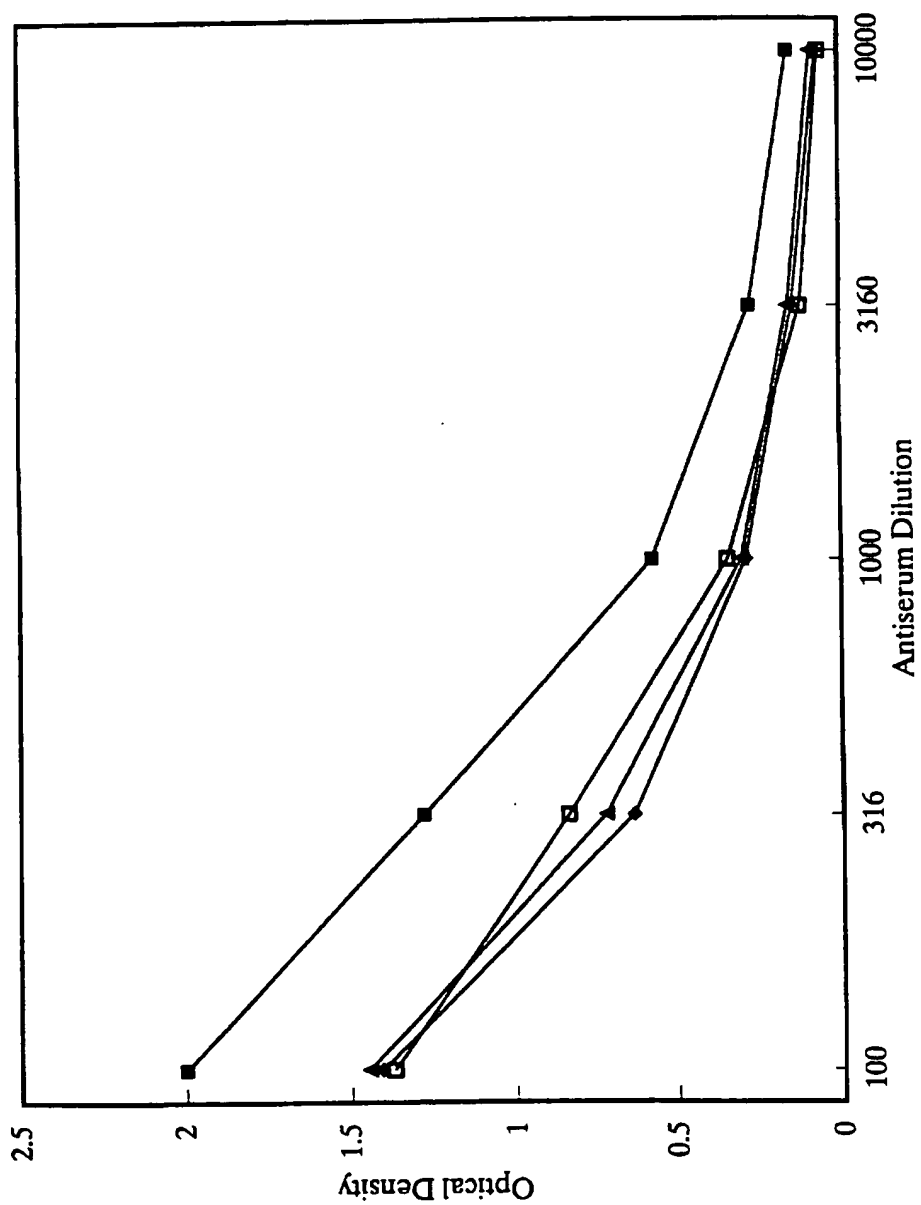


Fig. 9

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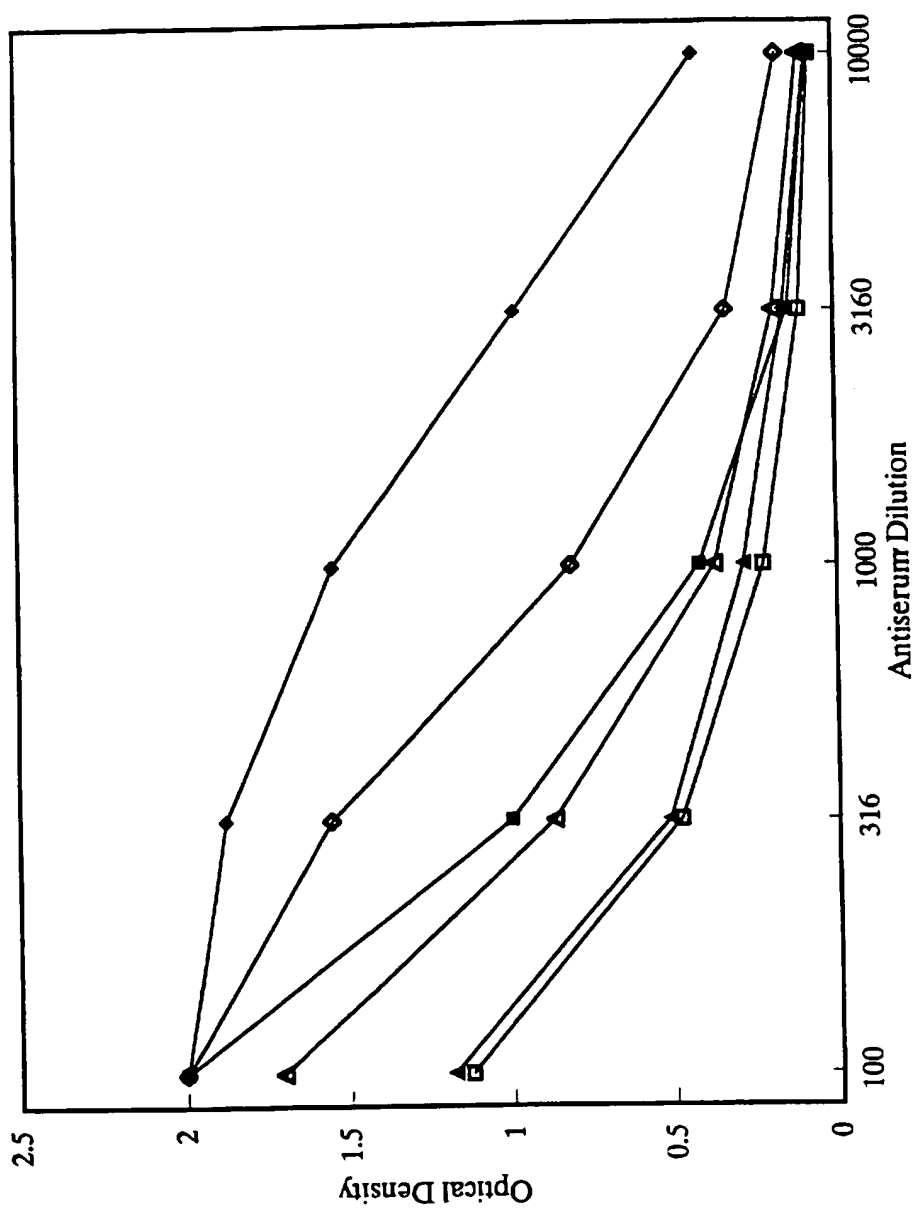


Fig. 10

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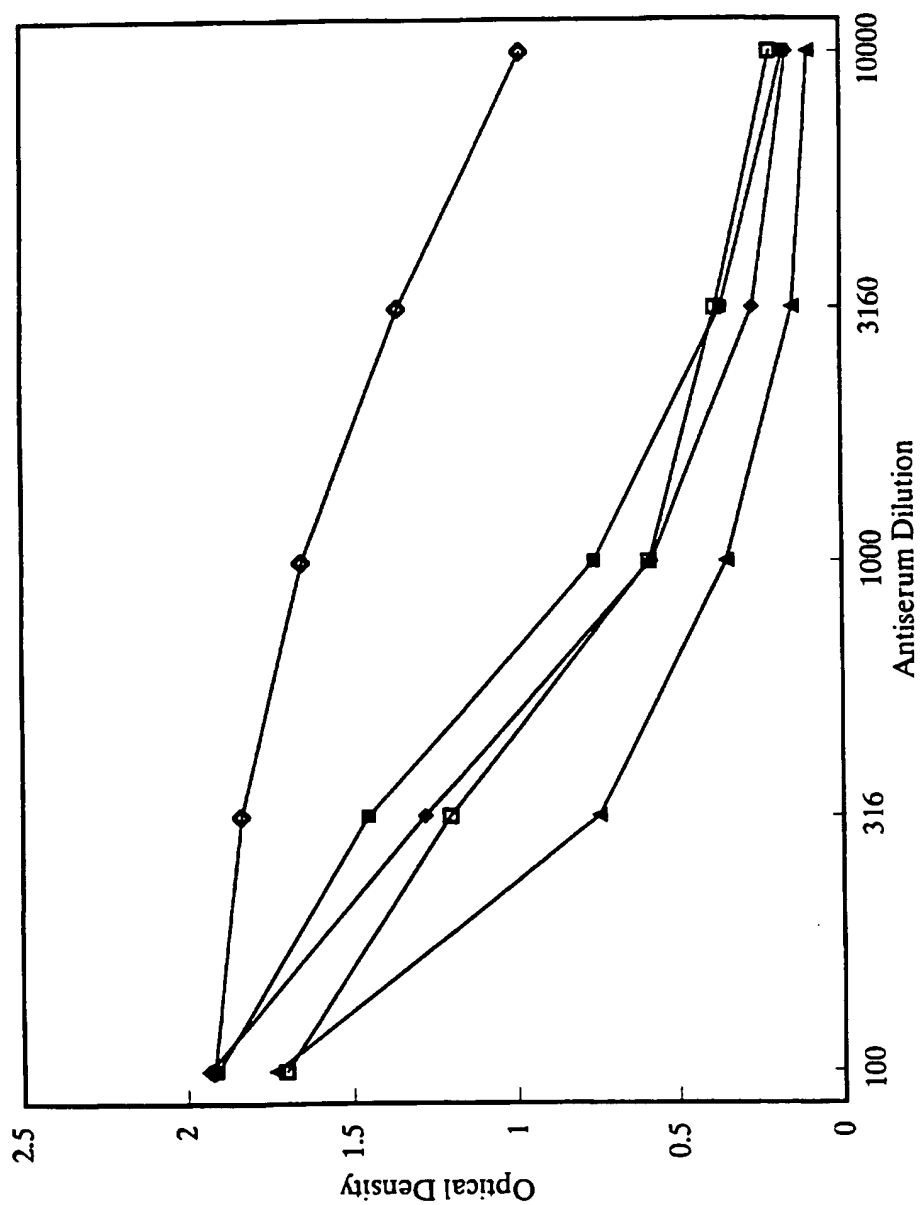


Fig. 11

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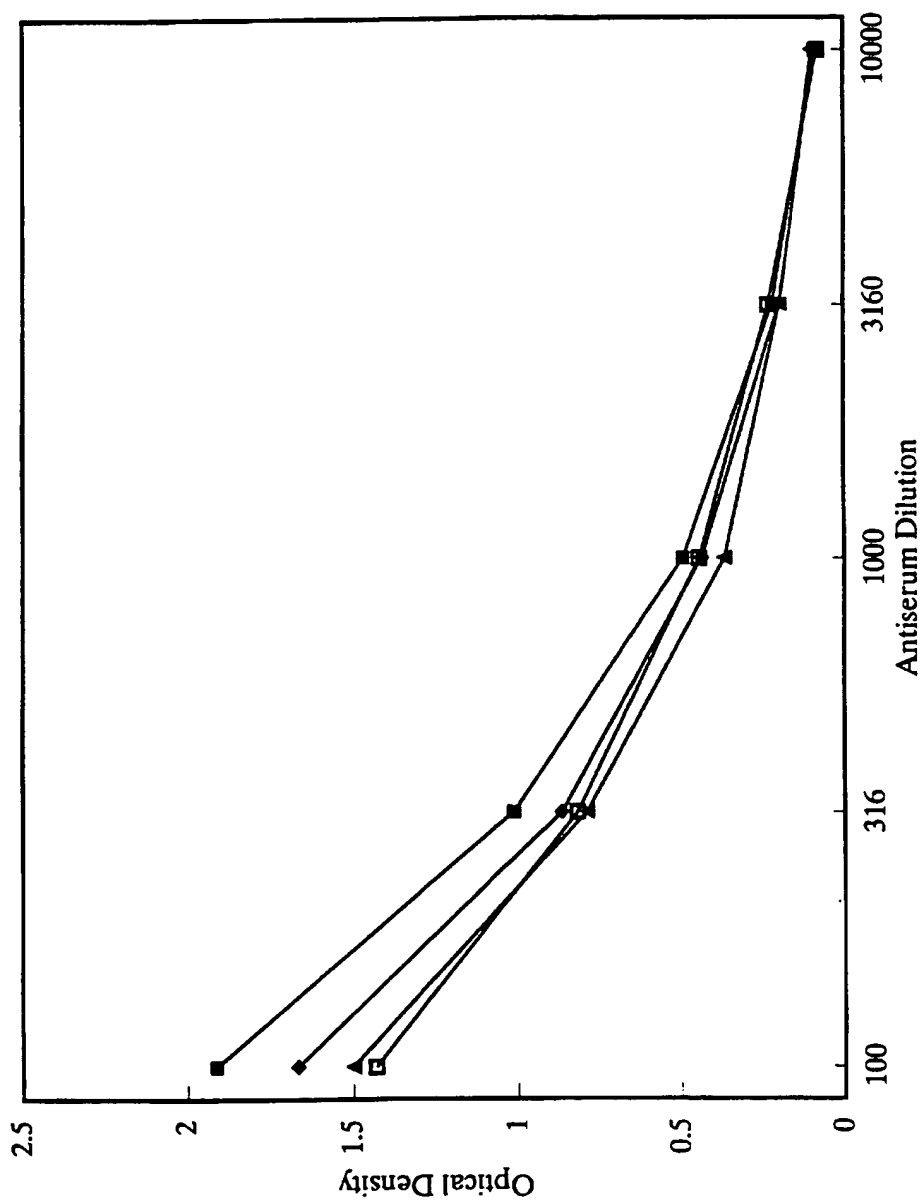


Fig. 12

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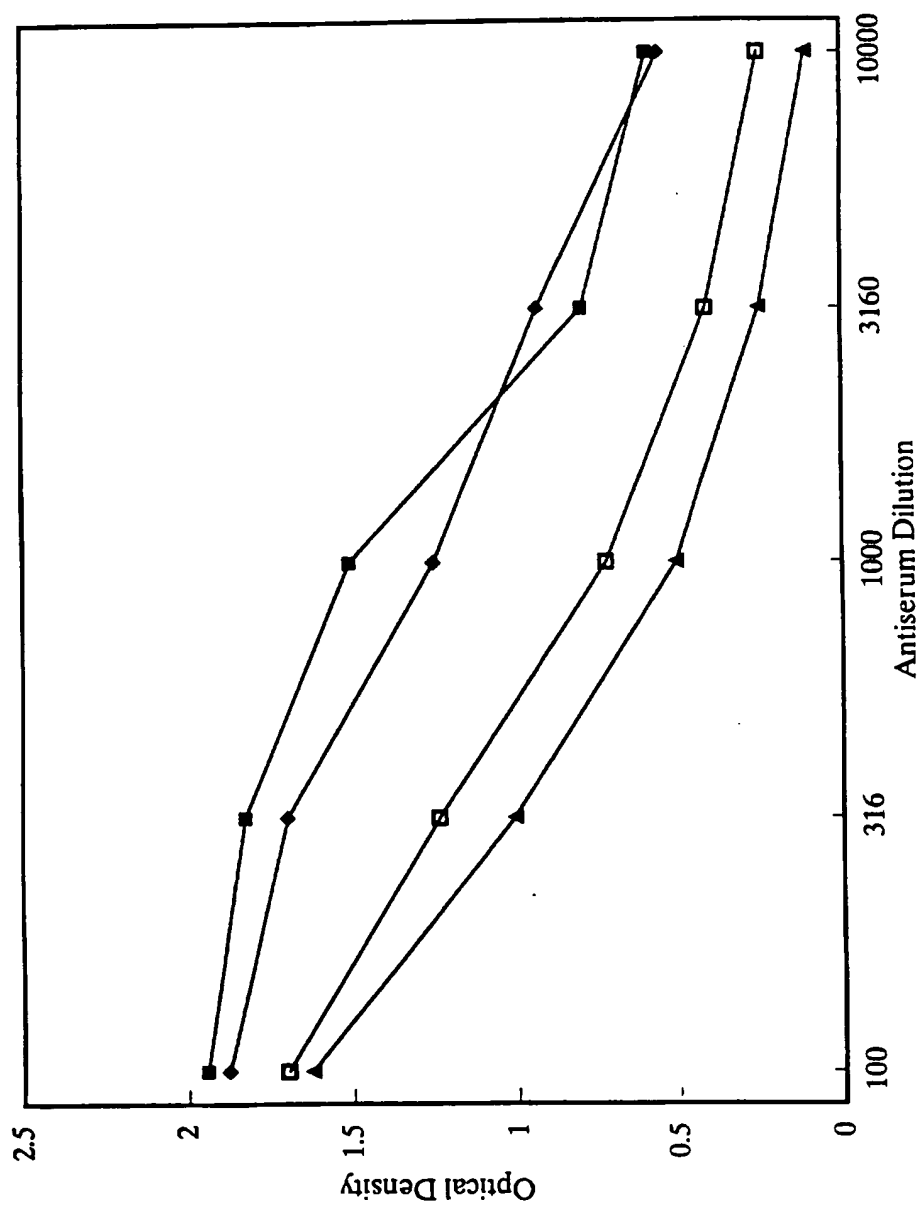


Fig. 13

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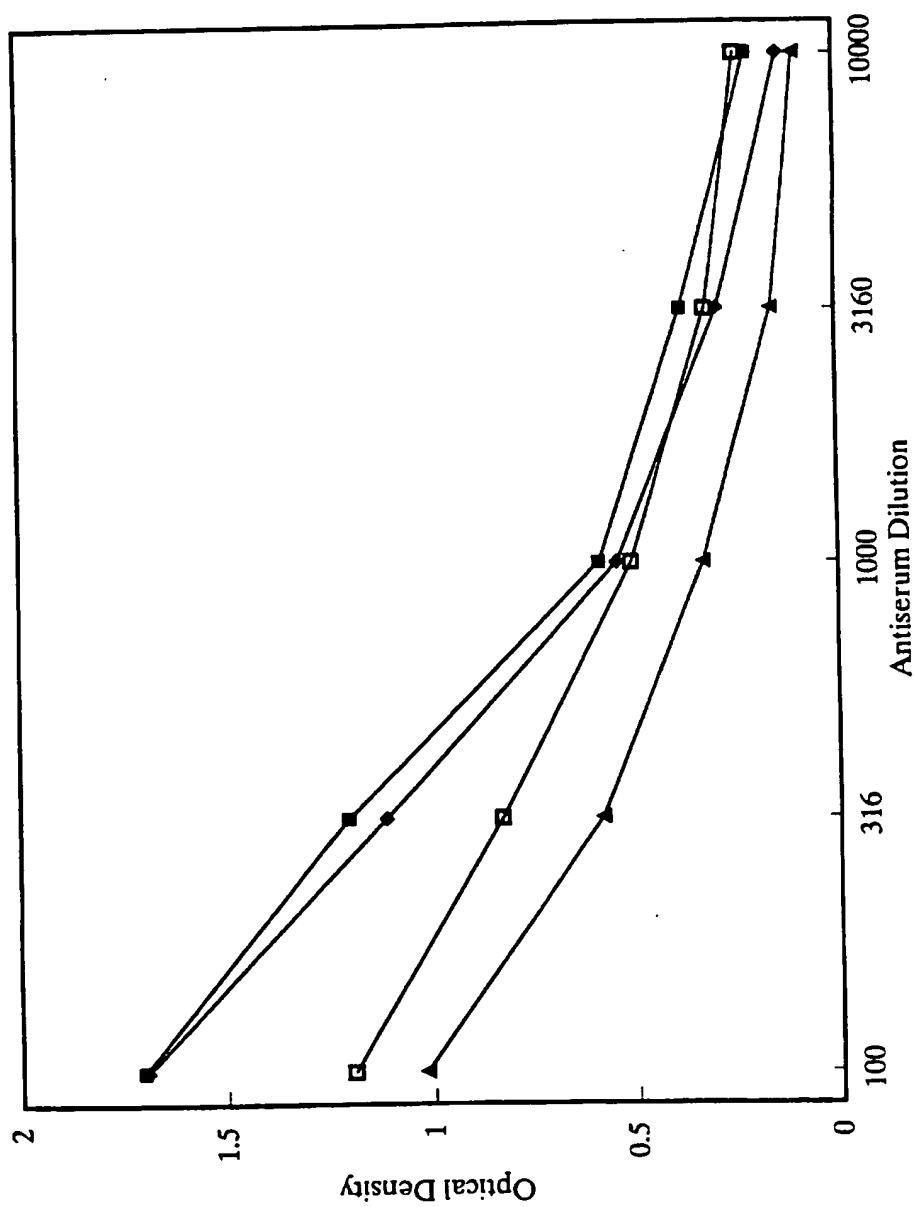


Fig. 14

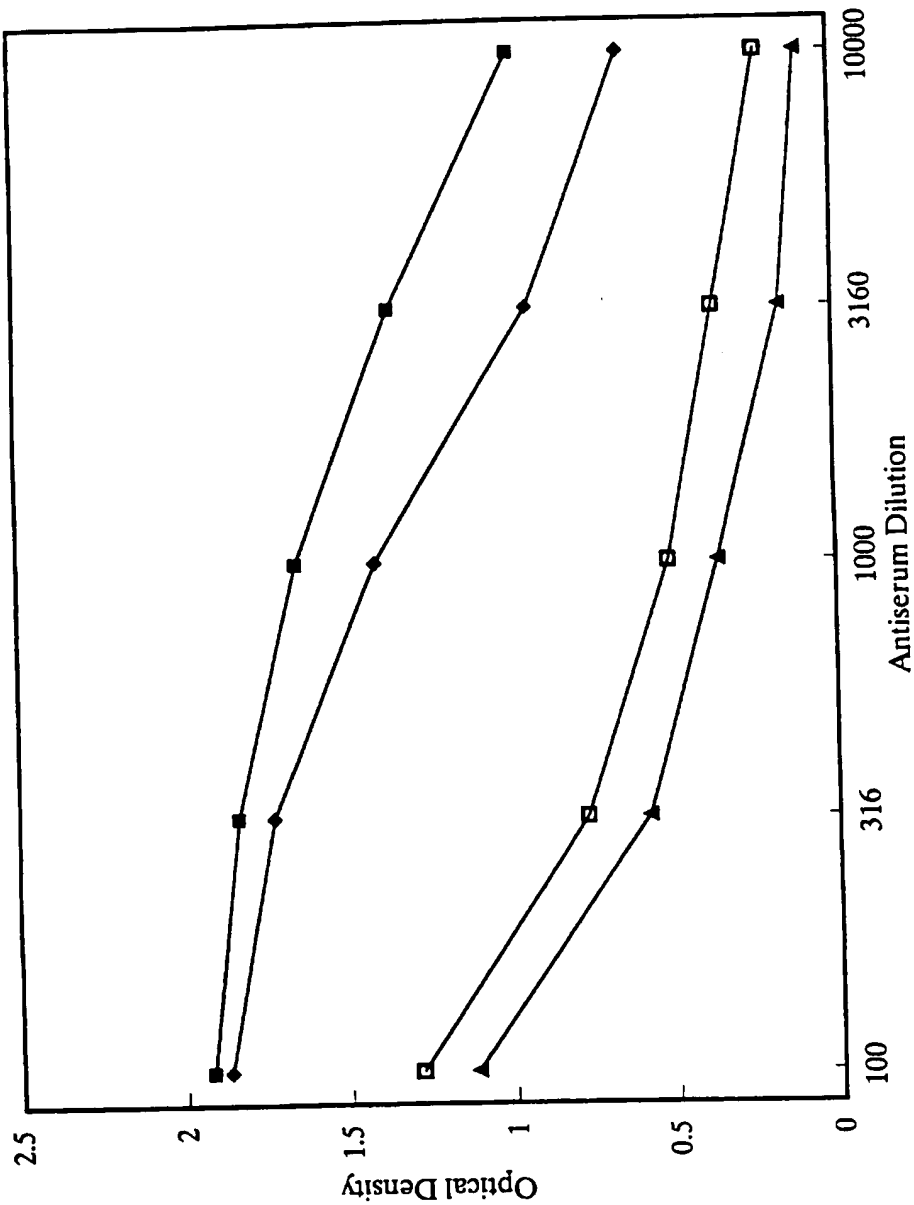


Fig. 15

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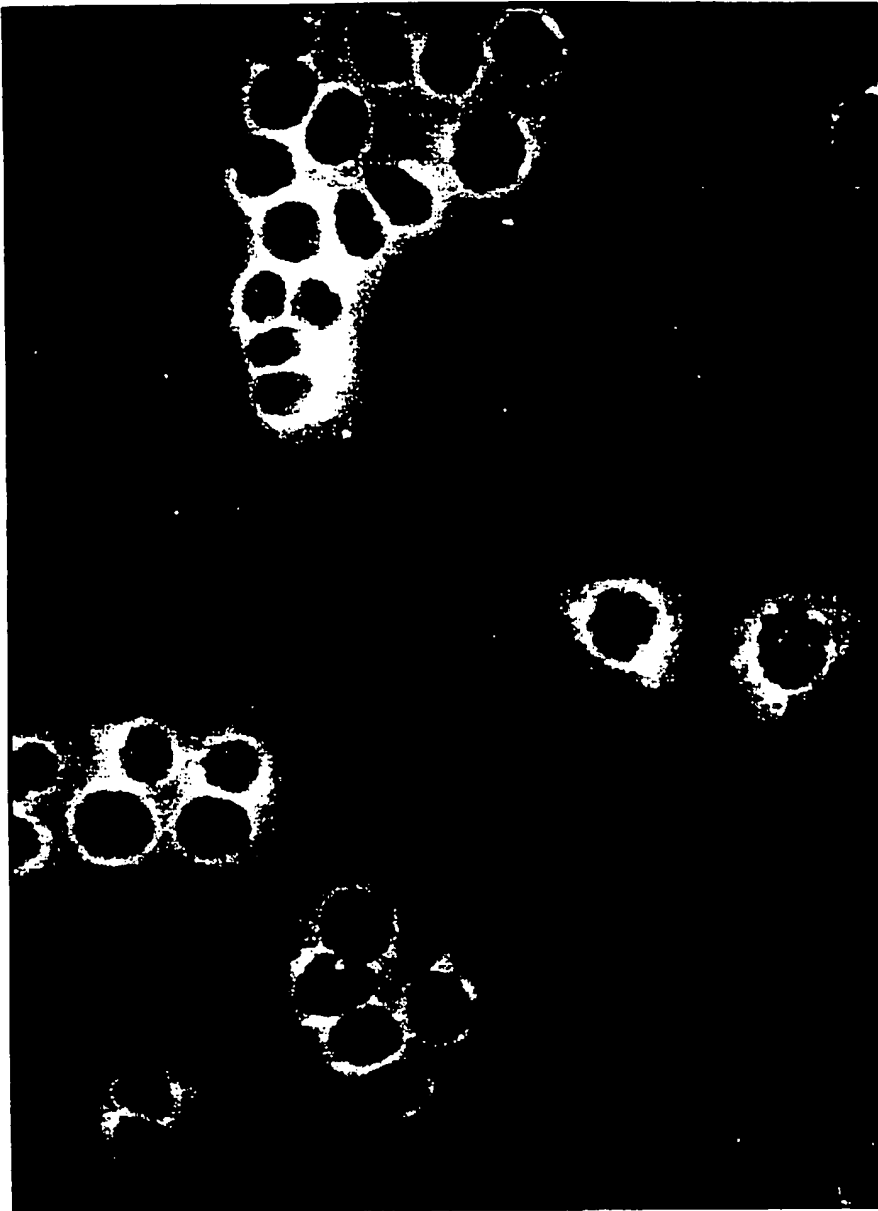


FIG. 16A

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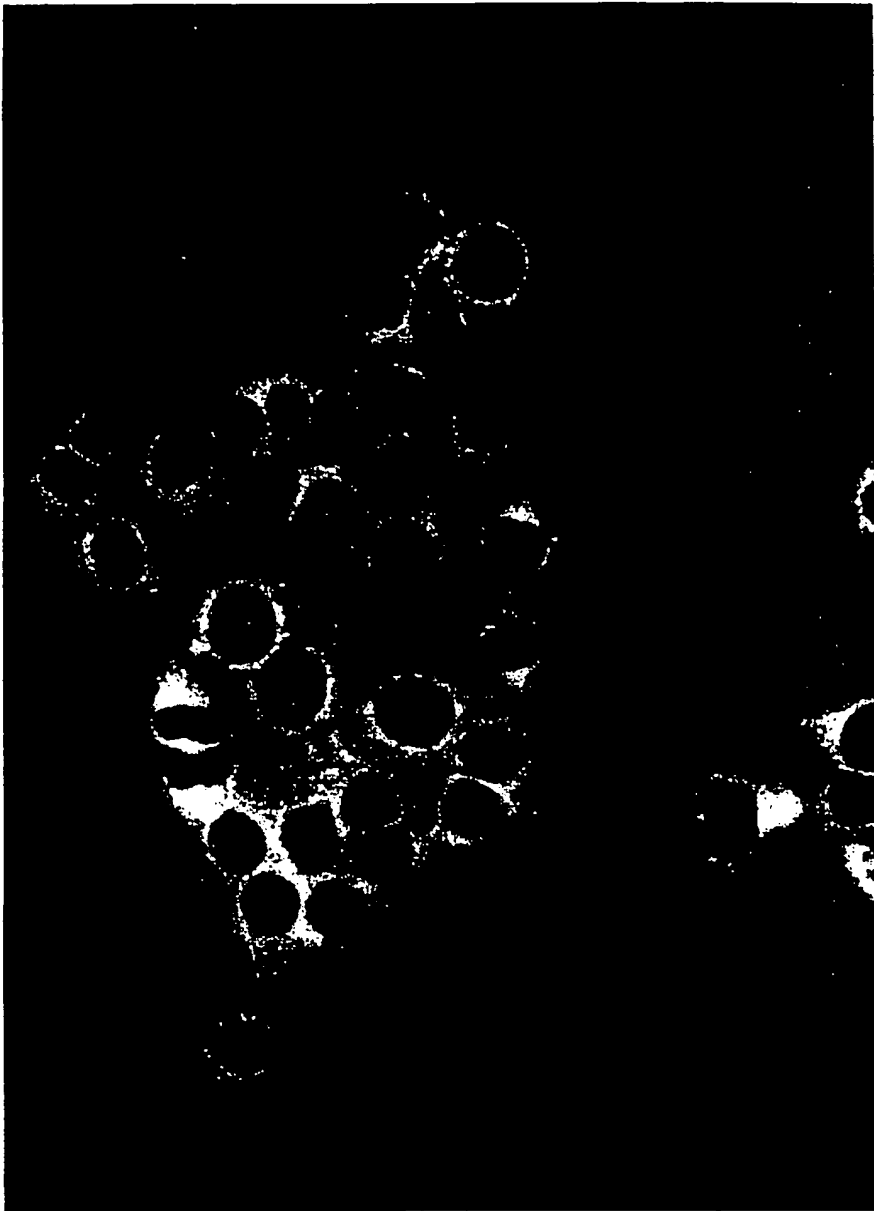


FIG. 16B

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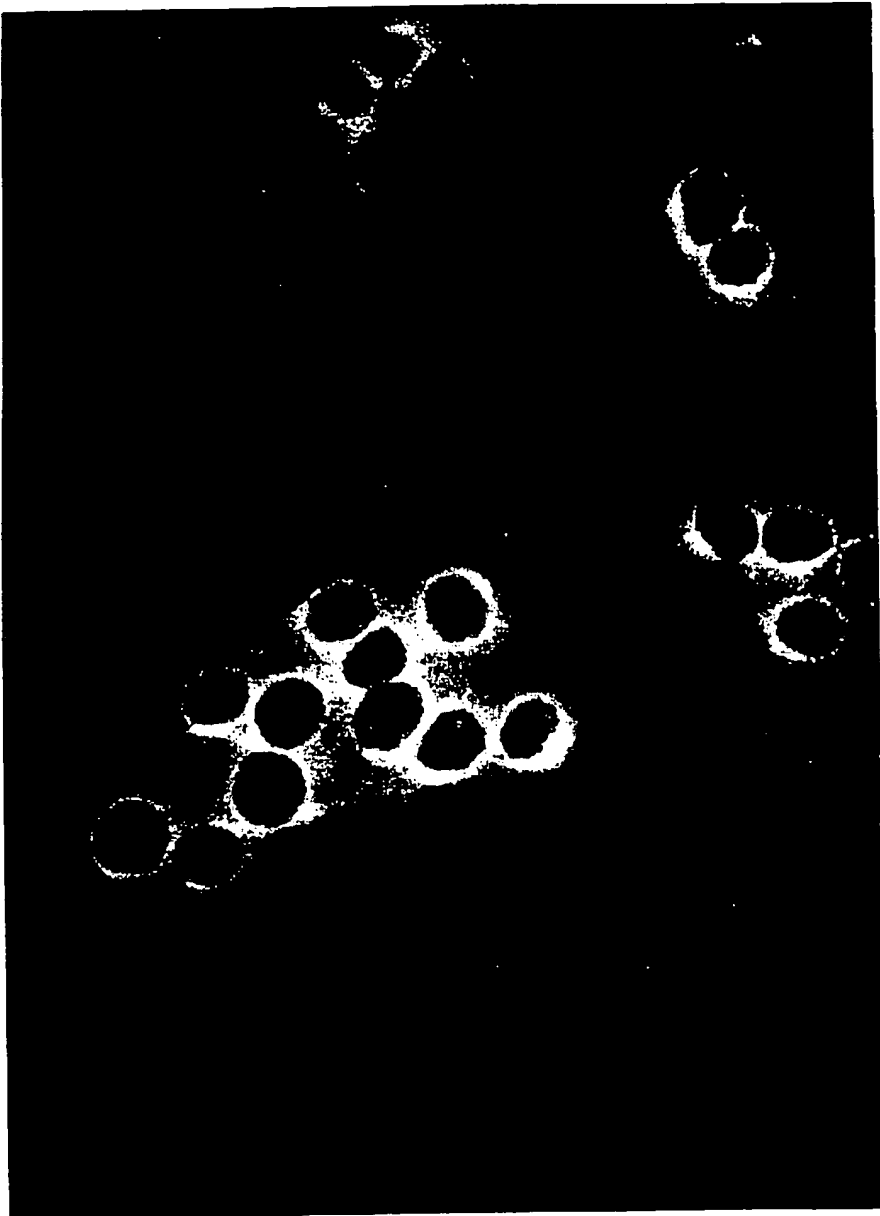


FIG. 16C

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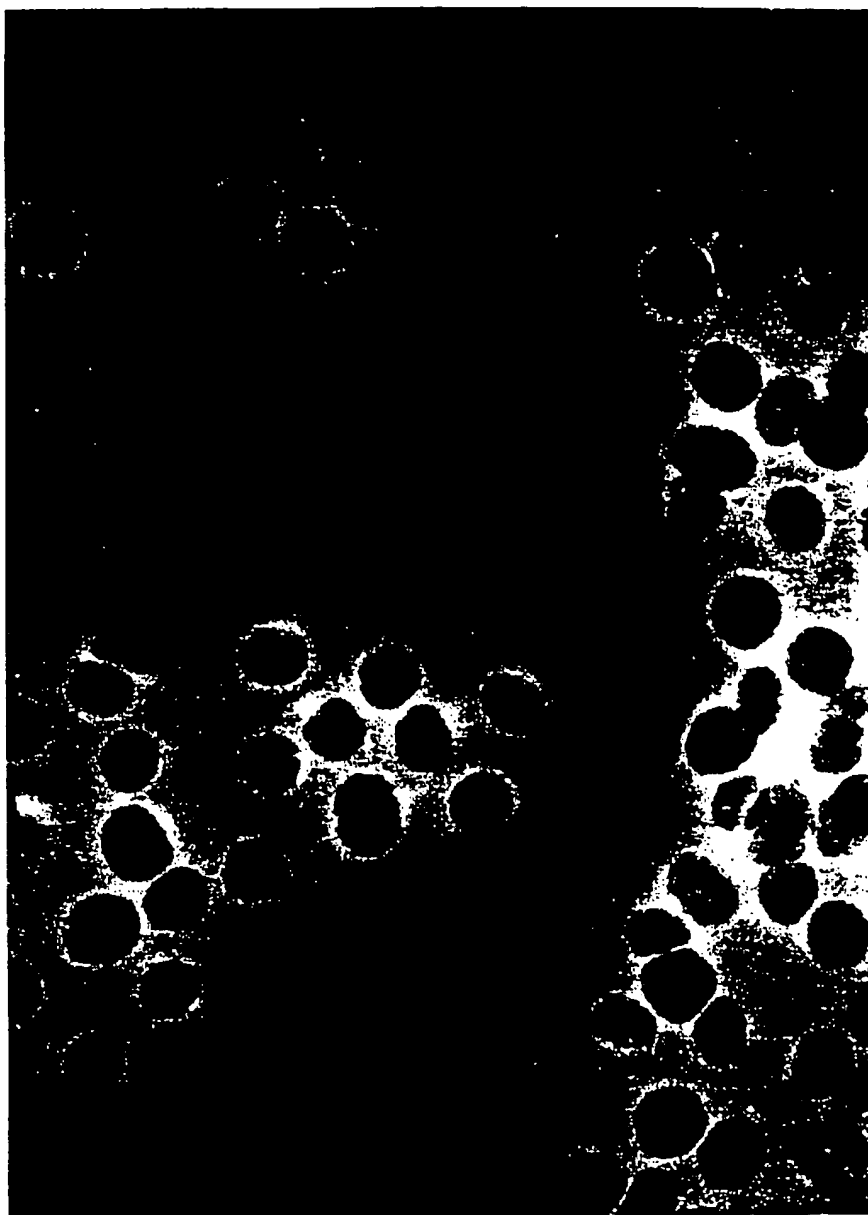


FIG. 16D

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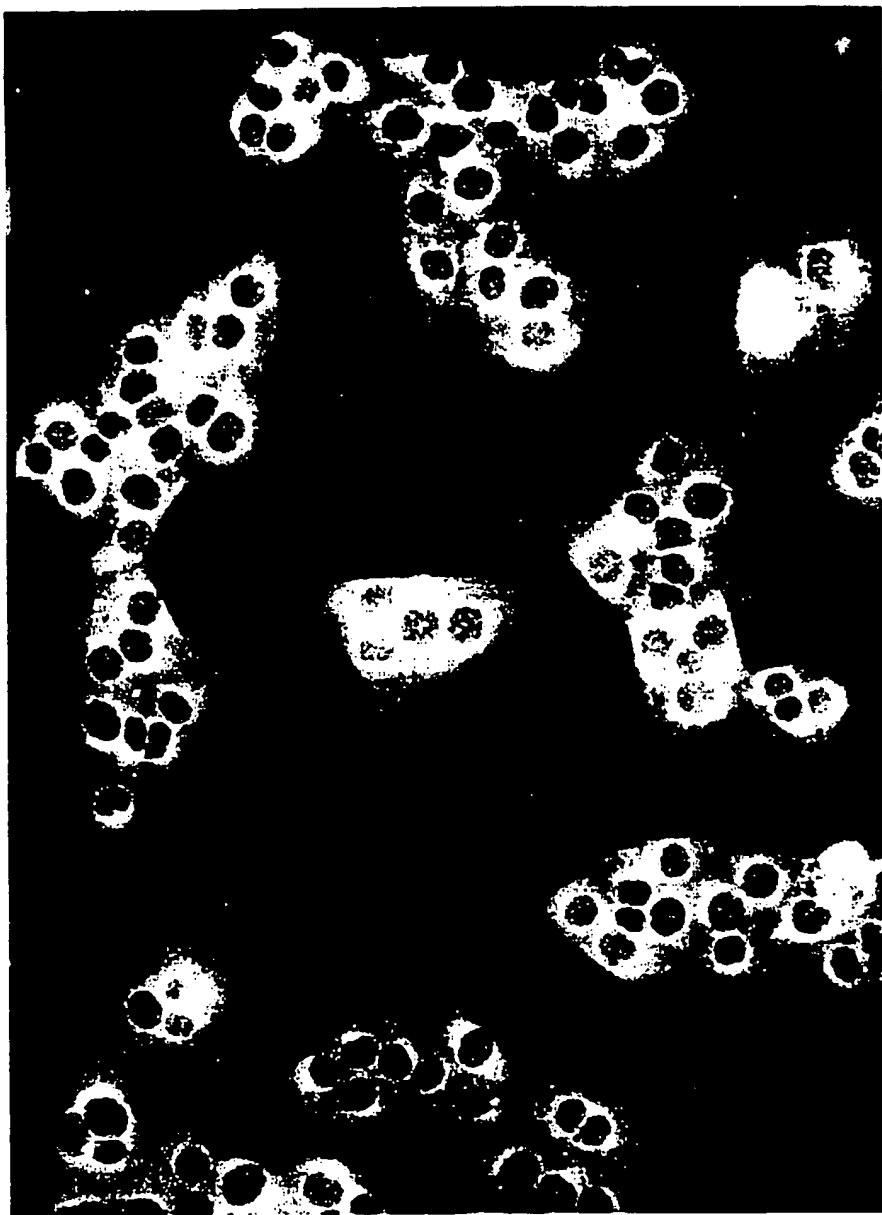


FIG. 16E

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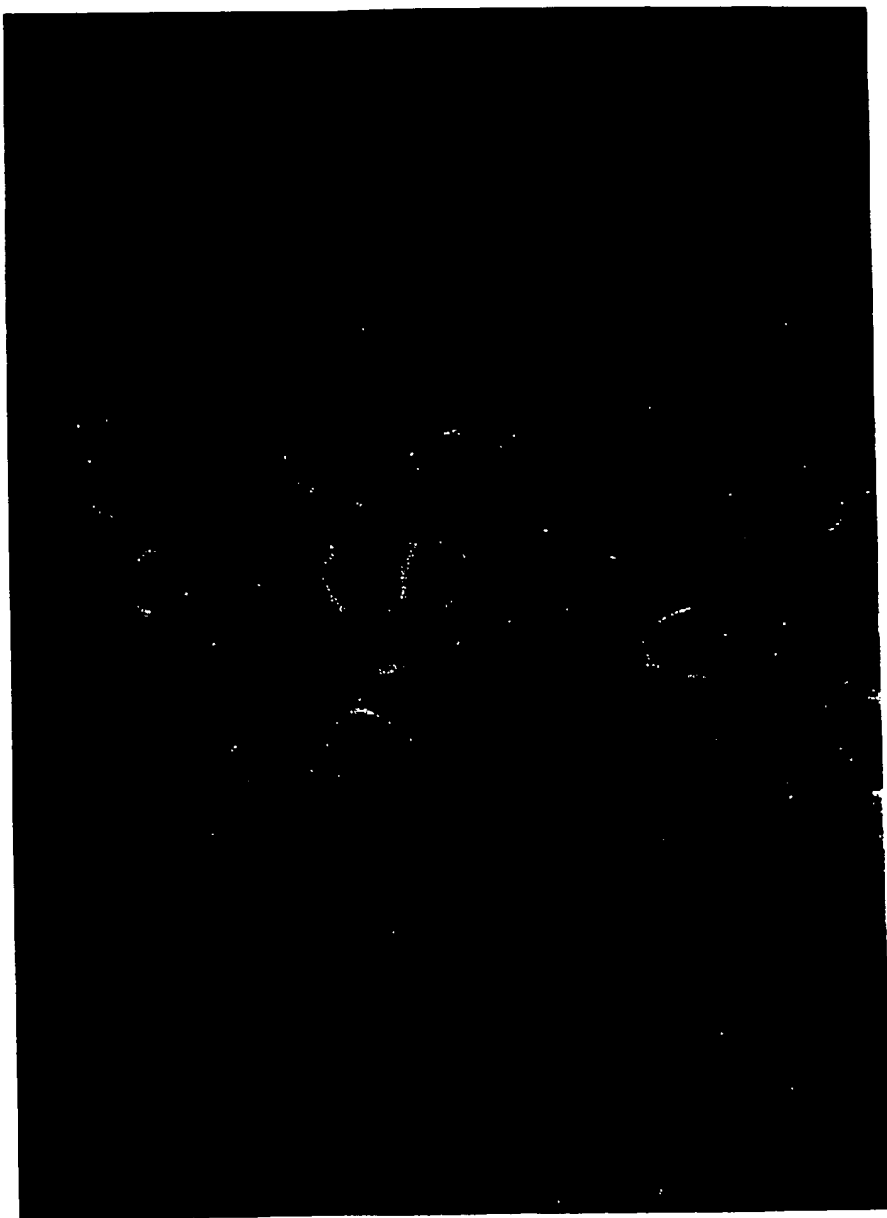


FIG. 16F

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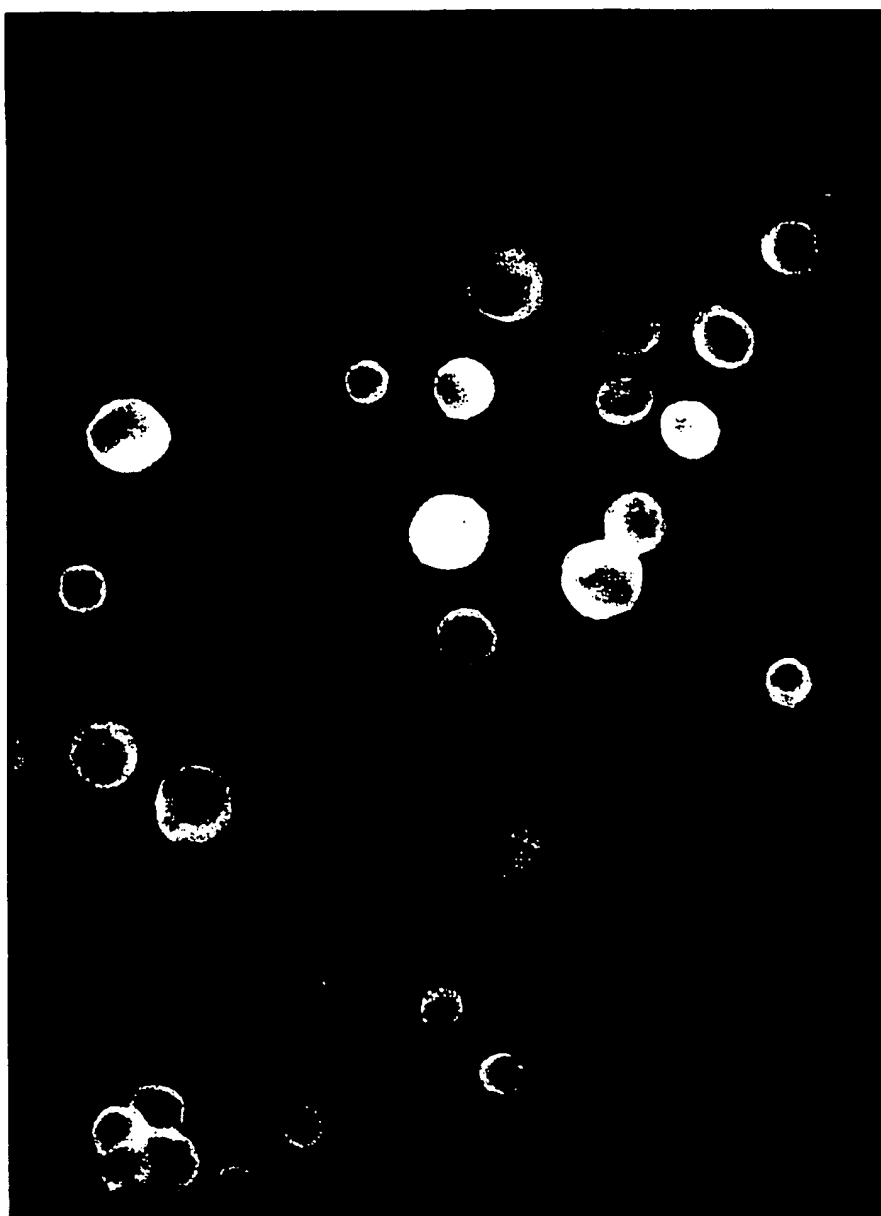


FIG. 16G

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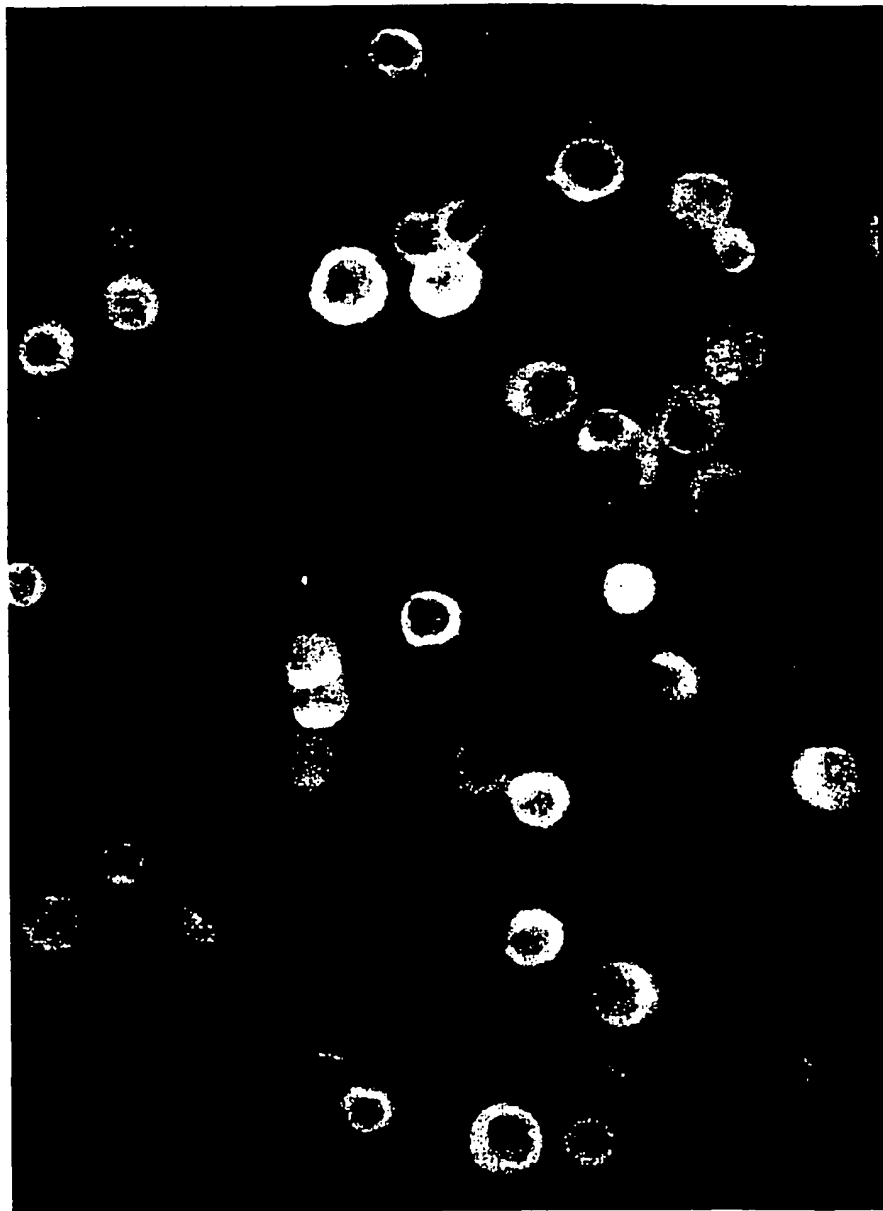


FIG. 16H

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FIG. 16I

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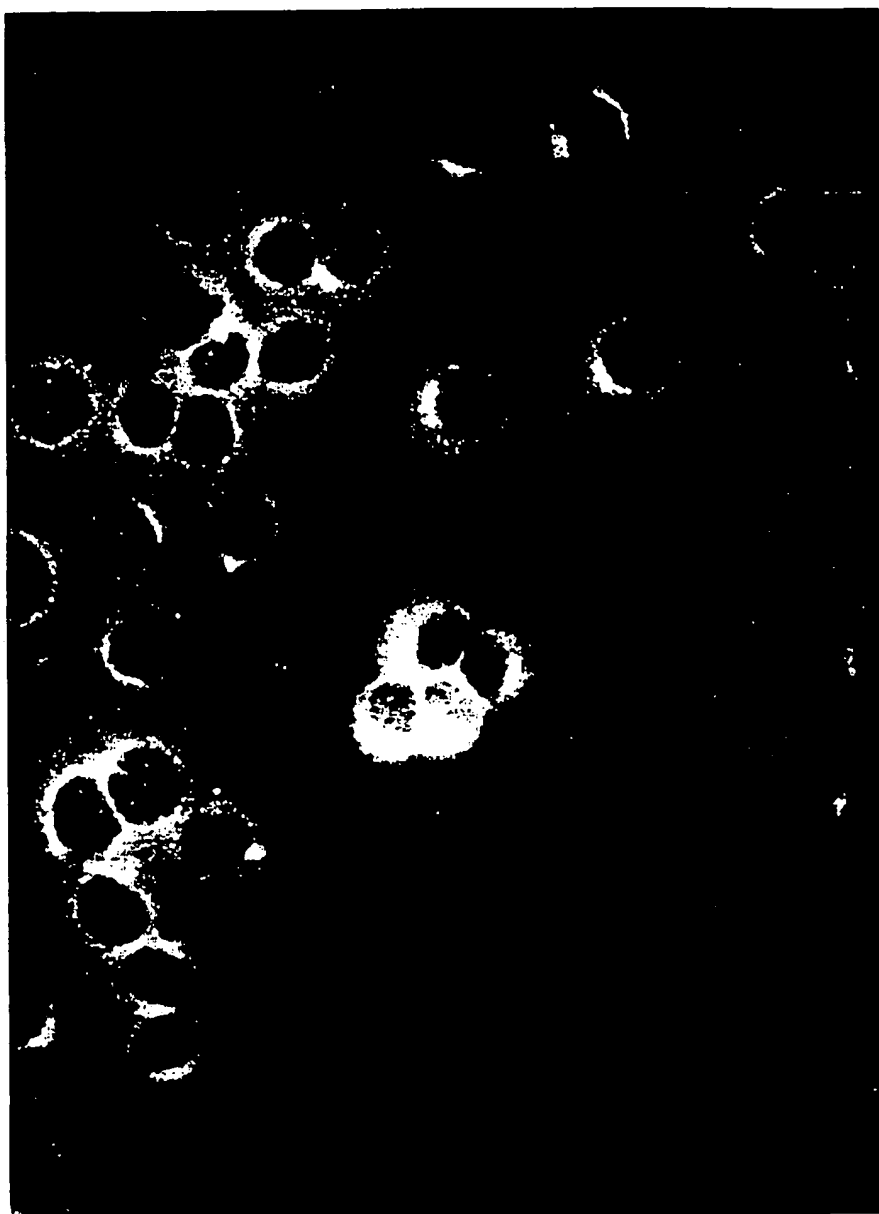


FIG. 16J

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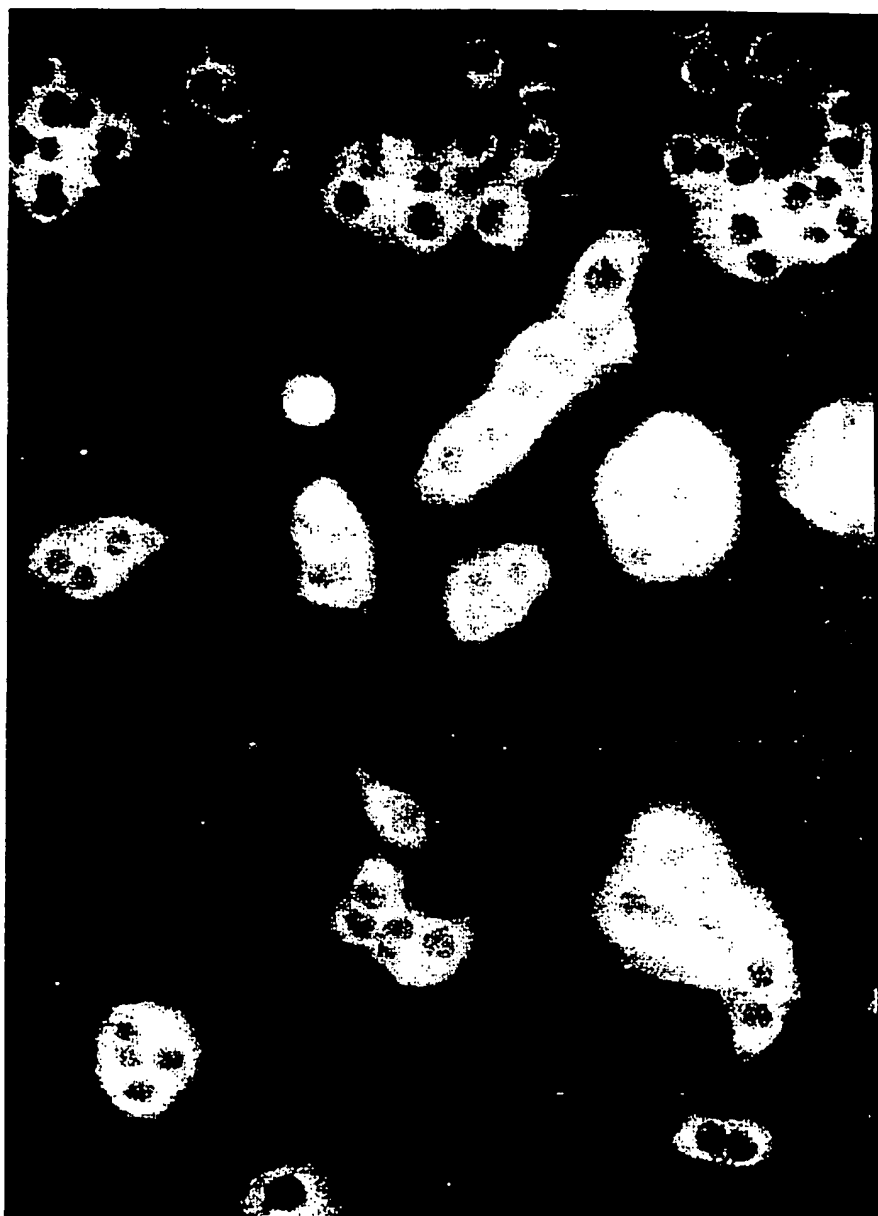


FIG. 16K

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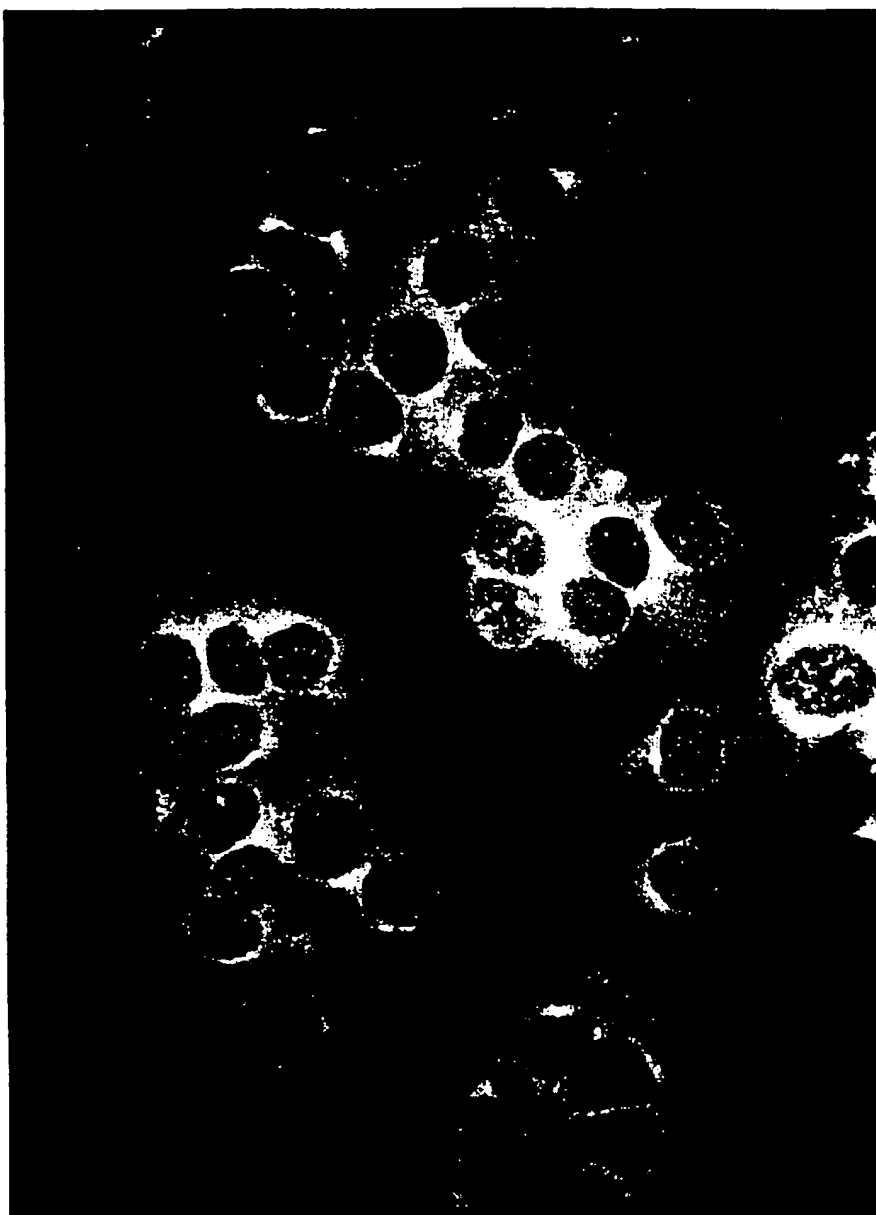


FIG. 16L

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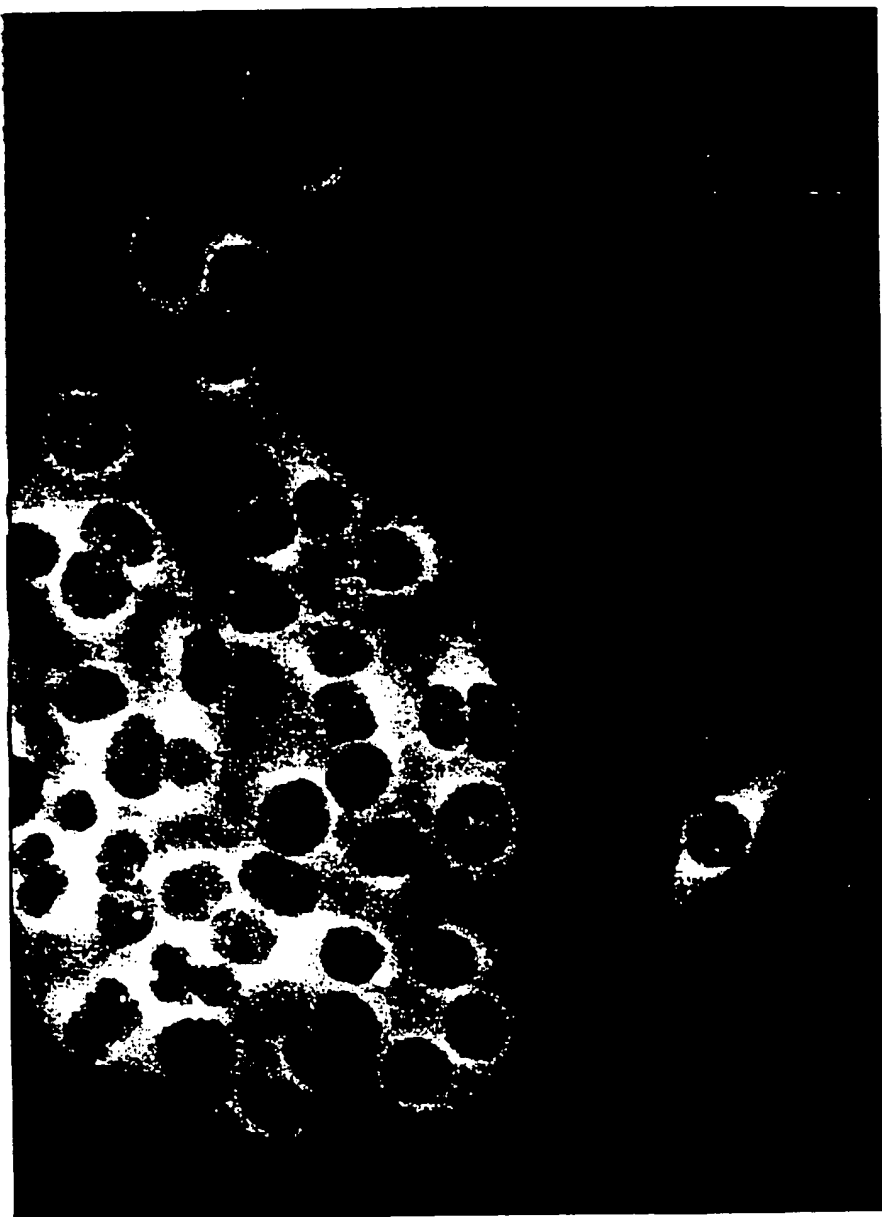


FIG. 16M

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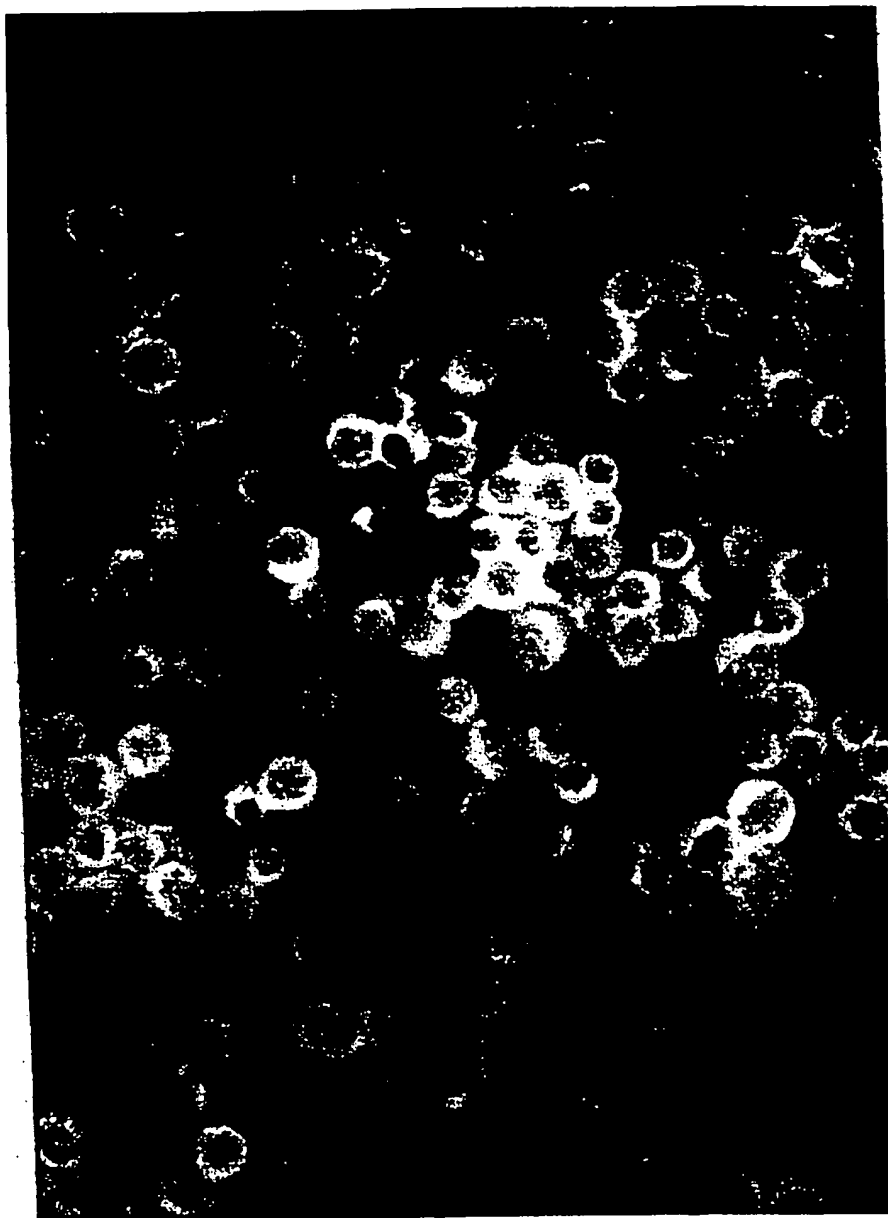


FIG. 16N



FIG. 160

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Western Immunoblots

PC-12 Lysate and
Dopamine Receptor Antisera

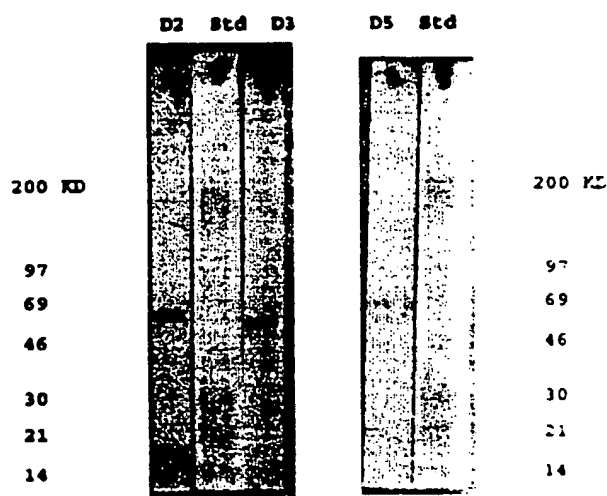


Fig. 17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/11127

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 7/06, 7/08, 14/705, 16/28

US CL : 530/326, 327, 328, 387.1, 388.2, 389.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/326, 327, 328, 387.1, 388.2, 389.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Registry, CAS, WPIDS, SwissProt31, PIR45, Geneseq

Search terms: dopamine receptor, antibody, peptide

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 347, issued 06 September 1990, Q.-Y. Zhou et al., "Cloning and expression of human and rat D1 dopamine receptors", pages 76-80, see entire document.	1-5
Y	Nature, Volume 347, issued 06 September 1990, R.K. Sunahara et al., "Human dopamine D1 receptor encoded by an intronless gene on chromosome 5", pages 80-83, see entire document.	1-5
Y	Nature, Volume 347, issued 06 September 1990, A. Dearry et al., "Molecular cloning and expression of the gene for a human D1 dopamine receptor", pages 72-76, see entire document.	1-5

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A		document defining the general state of the art which is not considered to be of particular relevance
* E		earlier document published on or after the international filing date
* L		document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
* O		document referring to an oral disclosure, use, exhibition or other means
* P		document published prior to the international filing date but later than the priority date claimed
	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	* A	document member of the same patent family

Date of the actual completion of the international search

21 NOVEMBER 1995

Date of mailing of the international search report

06 DEC 1995

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11127

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The EMBO Journal, Volume 8, Number 13, issued 1989, R. Dal Toso et al., "The dopamine D2 receptor: two molecular forms generated by alternative splicing", pages 4025-4034, see entire document.	1-5
Y	Proceedings of the National Academy of Science, Volume 86, issued December 1989, D.K. Grandy et al., "Cloning of the cDNA and gene for a human D2 dopamine receptor", pages 9762-9766, see entire document.	1-5
Y	Molecular Pharmacology, Volume 37, Number 1, issued January 1990, T.M. Stormann et al., "Molecular cloning and expression of a dopamine D2 receptor from human retina", pages 1-6, see entire document.	1-5
Y	Comptes Rendue Academie Science, Paris, Volume 311, Series III, issued 1990, B. Giros et al., "Clonage du gene du recepteur dopaminergique D3 humain et identification de son chromosome, pages 501-508, see entire document.	1-5
Y	Nature, Volume 350, issued 18 April 1991, H.H.M. Van Tol et al., "Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine", pages 610-614, see entire document.	1-5
Y	Nature, Volume 350, issued 18 April 1991, R.K. Sunahara et al., "Cloning the of the gene for a human dopamine D5 receptor with higher affinity for dopamine than D1", pages 614-619, see entire document.	1-5
Y	Proceedings of the National Academy of Science, Volume 88, issued October 1991, D.K. Grandy et al., "Multiple human D5 dopamine receptor genes: a functional receptor and two pseudogenes", pages 9175-9179, see entire document.	1-5
Y	Biochemical Society Transactions, Volume 19, issued 1991, P.L. Chazot et al., "Site-specific antibodies as probes of the structure and function of the brain D2 dopamine receptor", page 143S, see entire document.	1-5
Y	European Journal of Biochemistry, Volume 206, issued 1992, M.J. Plug et al., "An anti-peptide antibody that recognizes the dopamine D2 receptor from bovine striatum", pages 123-130, see entire document.	1-5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/11127

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemical Journal, Volume 289, issued 1993, P.L. Chazot et al., "Antisera specific for D2 dopamine receptors", pages 789-794, see entire document.	1-5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11127

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☒
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11127

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1 and 3, drawn to purified peptide analogs and to purified acetylated peptide analogs for D1, D2, D3, D4, and D5 dopamine receptors.

Group II, claim(s) 2, 4, and 5, drawn to monoclonal and polyclonal antibodies raised to purified peptide analogs for D1, D2, D3, D4, and D5 dopamine receptor.

The inventions listed as Groups do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons. The products claimed are peptide analogs for dopamine receptor subtypes (Group I), and antibodies made to said analogs (Group II). The products are distinct because they are made by different methods, have different structures, and have distinct functional properties. The peptide analog corresponds to a portion of the dopamine receptor, which serves to stimulate a cellular activity, and serves as an antigen to elicit the production of antibodies. The antibody is an immunoglobulin molecule and functions to bind its antigen. This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

Group I: Species A - peptide or acetylated peptide analog for D1 (claims 1 and 3)

Species B - peptide or acetylated peptide analog for D2 (claims 1 and 3)

Species C - peptide or acetylated peptide analog for D3 (claims 1 and 3)

Species D - peptide or acetylated peptide analog for D4 (claims 1 and 3)

Species E - peptide or acetylated peptide analog for D5 (claims 1 and 3)

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons. The peptide analogs are distinct from each other because they are from different subtypes of dopamine receptors. Although applicants disclose that the receptor subtypes are homologous, they do have different amino acid sequences, different structures, and different functions. For example, it is known in the art that certain dopamine receptor subtypes stimulate adenylyl cyclase, while others inhibit the activity of the enzyme. Currently, no claims are generic.

Group II: Species A - an antibody raised to a peptide analog for D1 (claims 2, 4, and 5)

Species B - an antibody raised to a peptide analog for D2 (claims 2, 4, and 5)

Species C - an antibody raised to a peptide analog for D3 (claims 2, 4, and 5)

Species D - an antibody raised to a peptide analog for D4 (claims 2, 4, and 5)

Species E - an antibody raised to a peptide analog for D5 (claims 2, 4, and 5)

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons. The peptide analogs are distinct from each other because they are from different subtypes of dopamine receptors, as noted above. Antibodies raised to a peptide analog for each receptor subtype are also different, since they bind to different receptor subtypes. Currently, no claims are generic.

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